Award Number: **W81XWH-09-1-0359**

TITLE: **Role of Stat3 and ErbB2 in Breast Cancer**

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REPORT DATE: **October 2012**

TYPE OF REPORT: **Annual Summary**

PREPARED FOR: **U.S. Army Medical Research and Materiel Command**
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: **Approved for public release; distribution unlimited**

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
1 October 2012	Annual Summary	15 Sep 2011-14 Sep 2012

4. TITLE AND SUBTITLE

Role of Stat3 and ErbB2 in Breast Cancer

5a. CONTRACT NUMBER
W81XWH-09-1-0359

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research &
Materiel Command Fort Detric, Ma
Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for public release; distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT : Activation of Stat3 has been demonstrated in breast and other cancers. However, the exact mechanism of its activation as well as the role of Stat3 in these tumors, remain to be determined. This study focuses on the identification of the upstream activators of Stat3 in confluent cells as well as the role of Stat3 in this setting. To explore the nature of cell to cell adhesion molecules responsible for Stat3 activation in breast cancer, we examined the effect of cadherin- 11 engagement using mouse Balb/c3T3 fibroblasts, which have high amounts of Cadherin -11, upon Stat3-ptyr705 levels and activity. Cadherin-11 knockdown experiments demonstrated that cadherin11 is required for Rac and Stat3 activation at high cell densities and is necessary for cell survival and migration.

We also further demonstrate for the first time that the mesenchymal, class I cadherin, N-cadherin whose activation correlates with metastasis, can also trigger a dramatic surge in Stat3. The fact that the mesenchymal cadherin-11 and N-cadherin may actually activate Stat3, although, contrary to the epithelial E-cadherin, they generally promote metastasis, may point to Stat3 as a central survival, rather than metastasis factor. Our resaults further demonstrate that the cadherin11/Stat3 axis may be necessary for transformation by Src, by being required to inhibit apoptosis triggered by the E2F transcription factor family, which is an indirect target of Src and a number of other oncogenes. Taken together, these data suggest that interference with cadherin-11 or N-cadherin function could induce apoptosis through Stat3 inhibition in metastatic cells specifically, a fact which could have important therapeutic implications.

15. SUBJECT TERMS
Stat3, ErBb2, Breast cancer

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U	UU	54	USAMRMC 19b. TELEPHONE NUMBER *(include area code)*

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

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Introduction

Cells in normal tissues or tumors have extensive opportunities for adhesion to their neighbors in a three-dimensional organization, and it recently became apparent that in the study of fundamental cellular processes it is important to take into account the effect of surrounding cells.

The best characterised classical type 1 cadherin is the epithelial (E)-cadherin (Ecad), which is responsible for the formation and maintenance of epithelial structures and is abundant in cultured cells of epithelial origin (*1*). Early results showed that continued expression of Ecad is required for cells to remain tightly associated within the epithelium, so that ***loss*** of ***Ecadherin*** function, including mislocalisation to the cytoplasm from the membrane, is associated with ***metastasis*** of invasive breast cancer (*2-4*). Over the years however, an increasing number of cadherins have been implicated in cancer as putative proto-oncogenic proteins, such as the "mesenchymal cadherins", N-cadherin (Ncad) and cadherin-11 which are often upregulated in cancer(*5*). Indeed, ***Ncad*** overexpression and engagement has been reported to be associated with a highly ***invasive*** phenotype and motility in mammary cell lines(*6-9*). In fact, normal squamous epithelial cell lines acquired migratory properties upon transfection with Ncad (*10*). Moreover, in certain tumour lines, such as MCF7 breast cancer cells which express Ecad and are not motile, transfected Ncad conferred a migratory phenotype, despite the presence of the endogenous Ecad(*11, 12*) .

Cadherin-11 (classical type II) was originally identified in mouse osteoblasts (*13*), but it was later found to be constitutively expressed in a variety of normal tissues of mesodermal origin, such as areas of the kidney and brain(*14*), as well as in cultured fibroblasts (*15*). Cadherin-11 was also shown to be elevated in a number of cancers where it correlates with a poor prognosis, and linked to breast cancer metastasis (*16, 17*). It is also expressed in prostate cancer and was shown to promote metastasis specifically to the bone (*18, 19*). It follows that examination of the mechanism of action of the different types of cadherins is of paramount importance in the study of cell to cell adhesion as well as metastasis.

Stat3 activity is required for transformation by a number of oncogenes and is found to be hyperactive in a number of cancers(*20*). The fact that a constitutively active form of Stat3 alone is sufficient to induce neoplastic transformation points to an etiological role for Stat3 in neoplasia. Stat3 is also activated by receptor tyrosine kinases, such as the epidermal growth factor receptor (EGFR) or platelet-derived growth factor receptor (PDGFR), as well as the non-receptor tyrosine kinase Src. We and others recently demonstrated that cell-cell adhesion causes a dramatic increase in the activity of Stat3 in breast carcinoma as well as normal epithelial cells and fibroblasts(*21-26*). We further demonstrated that E-cadherin engagement in mouse breast epithelial cells directly induces a dramatic increase in Rac and Stat3 activity, which constitutes a potent survival signal(*27*). This prompted us to examine the effect of mesenchymal cadherins whose expression correlates with metastasis, such as cadherin-11 and N-cadherin, upon Stat3 phosphorylation and activity. Our results indicate that engagement of cadherin-11 or N-cadherin can also trigger a dramatic surge in Rac/Cdc42 and Stat3 activity through upregulation of members of the IL6 family of cytokines, which is necessary for cell survival, proliferation and migration. The fact that cadherin-11 and N-cadherin may actually activate Stat3, although, contrary to E-cadherin, they generally promote metastasis, may point to Stat3 as a central survival, rather than metastasis factor. Hence, inhibition of cadherin-11 or N-cadherin could induce apoptosis through Stat3 inhibition in metastatic cells specifically, a fact which could have important therapeutic implications.

Body

Specific Aim 1. Examination of the upstream activators of Stat3 in confluent cells

Stat3 activation by Cadherin-11 requires Rac1/Cdc42

Early data demonstrated that cell to cell adhesion activates the Rac and Cdc42, Rho family GTPases(*28-30*). Therefore, to explore the potential effect of cadherin-11 engagement upon the levels of ***total*** Rac protein, detergent extracts from cells grown to different densities were blotted and probed for Rac. As shown in Fig. 1A, there was a sharp increase in ***total*** Rac protein levels with cell density, which could explain the increase in active Rac-GTP at high densities. Total Cdc42 levels mirrored Rac and increased at approximately the same time (Fig. 1A). Similar results were obtained with 10T½ cells (not shown). These results indicate that, in

addition to Rac and Cdc42 ***activity***, cell-cell adhesion also causes a dramatic increase in ***total*** Rac and Cdc42 protein levels.

To investigate whether the increase in Rac and Cdc42 protein levels with cell density is due to direct cadherin-11 engagement, Balb/c3T3 cells were plated in dishes coated with the two outermost extracellular domains of cadherin11, 11/EC, and Rac protein levels examined. As shown in Fig. 1B, plating on 11/EC-coated surfaces, besides leading to an increase in Stat3-ptyr705, also caused a dramatic increase in Rac protein levels. The above data taken together further demonstrate that direct cadherin-11 engagement is required for the cell-cell adhesion-mediated increase in the levels of these Rho family GTPases in Balb/c3T3 fibroblasts.

Since density was found to increase Rac levels in Balb/c3T3 cells, we next examined the effect of cadherin-11 knockdown, upon Rac1 protein levels (Fig. 1C & D). Balbc-shCad11 cells were plated to different densities and Rac levels examined and compared to the parental Balb/c3T3 cells by Western blotting analysis. As shown in Fig. 1E, Balbc-shCad11 cells had substantially lower Rac levels than the parental Balb/c3T3, indicating that cadherin-11 is required for Rac activation in confluent cultures.

Cell to cell adhesion triggers cytokine gene expression in mouse Balb/c 3T3 fibroblasts

We next examined the possibility that the cell density-mediated, Stat3 activation might occur through secretion of soluble factors. A quantitative RT-PCR array for mRNA of 86 cytokines was performed, by comparing sparsely growing cells to cells grown as dense cultures. The results revealed an increase in mRNA levels of a number of cytokines of the IL6 family, known to act through the common gp130 subunit, shared by a number of Stat3 activating cytokines, such as IL6, LIF, Ct1 and IL27 (32-fold for IL6 mRNA)(*31, 32*). To examine whether these cytokines are indeed required for the Stat3 activation observed in confluent cultures, the levels of gp130, the common subunit of the family were reduced through expression of shRNA with a retroviral vector. As shown in Fig. 2A, gp130 knockdown caused a dramatic reduction in Stat3-ptyr705 levels (Fig. 2B, lanes 1-3 *vs* 4-6), indicating that gp130 activation is at least partly responsible for the Stat3-ptyr705 increase.

Cadherin-11 engagement does not allow Erk1/2 activation by IL6

Besides Stat3, IL6 stimulation was shown to activate the Erk1/2 (Erk) kinase by triggering its phosphorylation at a TEY sequence (*31, 33*). However, p-Erk1/2 levels remain unaffected by cell density. By plating cells on surfaces coated with cadherin fragments, we demonstrated that direct cadherin engagement does not activate Erk, although Stat3-ptyr705 levels were dramatically increased. To solve this apparent paradox, we examined the ability of IL6 to activate Erk as a function of cell density. Balb/c3T3 cells were grown to 50% or 2 days postconfluence, serum-starved and following IL6 stimulation, cell extracts were probed for p-Erk or Stat3-ptyr705. As shown in Fig. 3A, at a confluence of 50%, IL6 addition caused a dramatic increase in both Stat3-ptyr705 (upper panel) and p-Erk ((lower panel), as expected, based on the published literature (*31*). As expected, cell density *per se* caused an increase in Stat3-ptyr705 levels **(**Fig. 3A, upper panel lanes 1-3 *vs* 4-6), and IL6 caused a further activation at both densities (lanes 1 vs 3 and 4 vs 6). Interestingly however, IL6 did not bring about an increase in p-Erk levels in densely growing cultures (Fig. 3A, lower panel, lanes 4 and 5), hinting at the possibility of a profound effect of confluence on the response of Balb/c3T3 cells to IL6 addition. These findings are in keeping with results from HC11 cells, which express E-cadherin(*34*). To investigate whether this might be due to cadherin function *per se*, the same experiment was conducted with the Balb-shCad11 cells, which are deficient in cadherin-11. As shown in Fig. 3B, in sharp contrast to the parental Balb/c3T3 cells, IL6 could stimulate Erk in densely growing, cadherin-11 deficient, Balb-shCad11 cells (Fig. 3B, lower panel, lanes 3 *vs* 4), indicating that it is indeed cadherin-11 engagement that prevents Erk activation by IL6.

N-cadherin activates Stat3

The type I classical cadherin, N-cadherin, was also shown to correlate with metastasis of tumor cells(*7*), therefore we examined its ability to activate Stat3. To this effect, we made use of the null, embryonal stem cells where E-cadherin was genetically ablated (Fig.4A) (*27*). These cells have very low background levels of Stat3, which might be due to the Leukemia inhibitory factor necessary for their growth(*27*). N-cadherin expression in

these cells caused an increase in Stat3-ptyr705 (Fig.4B), indicating that N-cadherin can also activate the Stat3 pathway in this cellular setting.

To further confirm the ability of N-cadherin to activate Stat3, we transfected a construct of Ncadherin-GFP in HEK-293 cells which express low levels of N-cadherin (Fig.4C, lane 3). As shown in Fig. 4C (lanes 1 and 2), transient N-cadherin expression caused a dramatic increase in Stat3-ptyr705 levels, further confirming that N-cadherin also can activate Stat3 following transient expression. Taken together, the above data indicate that N-cadherin, a cadherin which, contrary to E-cadherin correlates with metastasis of epithelial cells can activate Stat3.

Cadherin-11 is required for transformation by vSrc:

Research from a number of labs previously demonstrated that vSrc negatively affects the function of cadherins(*35-38*). Surprisingly however, despite the fact that vSrc may reduce cadherin-mediated adhesion, previous results in our lab showed that cell density still caused a significant increase in Stat3 activity in vSrc transformed cells, indicating that residual amounts of cadherin may be important in transformation by Src (*21*). Moreover, Stat3 inhibition induced apoptosis in transformed cells which was more pronounced at high cell densities(*39*). This prompted us to look for a potential positive role of cadherins in Src transformation.

A large number of oncogenes such as Src are known to activate transcription factors such as the E2F family, which control the expression of genes involved in cell division. Interestingly, E2F is also a potent ***apoptosis*** inducer, hence the high demand of transformed cells for survival signals. However, Src also activates anti-apoptotic signals such as Stat3 and PI3 kinase, so that the net effect of Src expression is cell division and neoplasia. Src inhibition therefore would be expected to return a cell to a normal phenotype, without inducing its death, since it would remove both the E2F (apoptotic) and Stat3 (anti-apoptotic) signals. Inhibition of Stat3 however would be expected to induce apoptosis in Src-transformed cells preferentially, due to their higher E2F activity levels. Since cadherin engagement was shown to be a potent Stat3 activator, we examined the effect of cadherin-11 knockdown in Balb/c3T3 fibroblasts transformed by Src. Our results demonstrated that in Src-transformed cells, cadherin-11 knockdown is a powerful way to induce Stat3 inhibition and apoptosis, which shows the importance of the cadherin11/Stat3 axis in apoptosis inhibition in Src-transformed cells. Cadherin-11 knockdown also caused a decrease in transformation parameters such as focus formation on plastic and anchorage-independent growth (Fig.5), indicating that cadherin-11 is needed for Src-transformation as well. Most importantly, since it was shown that cadherin-11 is present in metastatic cells specifically, inhibition of cadherin-11 is expected to induce apoptosis in transformed, metastatic cells, while normal cells, expressing E-cadherin would be spared. Cadherin-11 knockdown in normal cells would not induce apoptosis, due to their lower E2F levels.

Activation of Stat3 by cadherin engagement was previously shown to require the gp130 receptor. We now demonstrated that gp130 is also required for transformation by Src, possibly to inhibit apoptosis induced by the high E2F activity. Therefore, despite its presumed structural role, the cadherin/Rac/gp130/Stat3 axis is a critical determinant in Src transformation. This work is underway.

This work led to an interesting spinoff:

Our observation that Stat3 activity is increased by cell confluence led us to examine the effect of Stat3 upon gap junctional, intercellular communication (GJIC), which is dependent upon cadherin engagement:

Gap junctions are channels that connect the cytoplasm of adjacent cells. Gap junctional, intercellular communication (GJIC) is blocked in cells transformed by oncogenes such as activated Src. One of the Src effector pathways leading to GJIC suppression and transformation is the Ras/Raf/Mek/Erk, so that inhibition of this pathway in vSrc-transformed cells restores GJIC. A distinct Src downstream effector required for neoplasia is the Signal Transducer and Activator of Transcription-3 (Stat3).

We previously examined the effect of Stat3 upon GJIC by electroporating the fluorescent dye, Lucifer yellow, into cells grown on two co-planar electrodes of electrically conductive, optically transparent, indium-tin oxide, followed by observation of the migration of the dye to the adjacent, nonelectroporated cells under fluorescence illumination. The results demonstrated that, in sharp contrast to inhibition of the Ras pathway,

Stat3 inhibition in Src-transformed, rat liver epithelial T51B cells through treatment with the CPA7, Stat3 inhibitor, or through infection with a retroviral vector expressing a Stat3-specific shRNA, does ***not*** restore GJIC. Furthermore, we examined the effect of Stat3 inhibition in normal cells with high GJIC. Strikingly, our results demonstrate that Stat3 inhibition ***eliminates*** GJIC in T51B cells possessing extensive communication(*40*). We now expressed a constitutively active form of Stat3, Stat3C in T51B cells, and in two lung cancer lines that still retain high GJIC. Our results demonstrate that Stat3C caused a dramatic increase in GJIC in both lines. Therefore Stat3, although it is generally growth promoting and in an activated form can act as an oncogene, its function is actually ***required*** for and increases junctional permeability. These results are in preparation.

I was invited to write a review on the effect of the Src oncogene and its effectors upon GJIC, for the Journal ***Anticancer research*** (Geletu *et al*, ***Anticancer research***, 2012).

Specific Aim 2: Examination of the role of Stat3 in confluent cultures: Effect of Stat3 upon p53

cav1 downregulation increases cadherin-11 levels in mouse Balb/c3T3 fibroblasts

Caveolin 1 (cav1), a 22 KDa membrane protein is the major protein responsible for the organization and maintenance of caveolae microdomains (*41, 42*)**.** Cav1 recruits many receptor and non-receptor tyrosine kinases and through binding to its scaffolding-domain, cav1 sequesters the kinases in an inactive form, thereby preventing their involvement in signaling pathways(*42*). We previously demonstrated that cav-1 downregulation activates Stat3, while cav1 overexpression downregulates Stat3 and induces a growth retardation or apoptosis. At the same time, normal growth and survival was restored by co-expression of the constitutively active form of Stat3, Stat3C. Given the close relationship between Stat3 and cadherin-11, we decided to examine the effect of cav1 upon cadherin-11 levels. Cav1 was downregulated using a cav1 shRNA retroviral construct in Balb/c3T3 cells. The results revealed that cav1 knockdown caused an increase in Cad11 levels, (Fig. 6). This finding argues for cav1 as a negative regulator of cadherin11, which may explain the increase in Stat3 upon cav1 downregulation observed.

Cadherin 11 or Rac1 downregulation increases cav1 levels

Cadherin-11 plays a very important role in regulating cellular motility. In fact, previous studies indicated that cadherin-11 is aberrantly expressed in cancer cells with an invasive phenotype and increased risk for metastasis (*19, 43*). My recent results (Geletu et al, in preparation), in agreement with a recent report (Li et al., 2011) also demonstrate that cadherin-11 promotes Rac1 activation and cell migration in metastatic breast cancer cells. On the other hand, cav1 is also part of a signalling pathway that leads to the ubiquitylation and degradation of Rac1(*44*). Therefore, we examined the effect of cadherin-11 and Rac1 upon cav1 level. Cadherin-11, or Rac1 were downregulated using shRNA expressing, retroviral constructs. As shown in Fig. 7 (A & B), cadherin-11 or Rac1 knockdown caused an increase in cav1 levels, at all densities examined. These results taken together demonstrate the presence of a potent, negative regulatory loop between cav1 on the one hand, and cadherin-11 and Rac on the other.

Specific Aim 3. Examination of the incidence of Stat3 activation, in conjunction with Rac1, Cav1, Cadherin11 and p53 levels in primary tumors, and correlation with the type of tumor, resistance to Herceptin, disease stage and outcome.

This part of the project is ongoing as a concerted effort by Oncologists, Pathologists and basic scientists. Our results so far showed that the automated quantitative analysis of p53, cyclin D1, Ki67 and pERK expression in breast carcinoma using the ARIOL system does not differ from expert pathologist scoring and correlates with clinico-pathological characteristics. These data were recently published (Cass *et al*, ***Cancers***, in press, September, 2012) and will now be expanded to a larger array that will include Rac, cadherin-11 and cav1.

Key Research Accomplishments

- **Stat3 activation by Cadherin-11 requires Rac1/Cdc42**
- **Cell to cell adhesion triggers cytokine gene expression in mouse Balb/c 3T3 fibroblasts**
- **Cadherin-11 engagement does not allow Erk1/2 activation by IL6**
- **N-cadherin also activates Stat3**
- **Cadherin-11 is required for transformation by vSrc**
- **cav1 downregulation increases cadherin-11 levels in fibroblasts**
- **Cadherin 11 and Rac1 downregulation increases cav1 level**

Reportable Outcomes

Award

Queen's University office of Research services postdoctoral travel award for participating in the inaugural Canadian Cancer Research Conference, November 27-30, 2011 in Toronto, Ontario.

Papers published or in press

Geletu M, Greer S, Arulanandam R, Tomai E and Raptis, L. Stat3 is a positive regulator of gap junctional intercellular communication in cultured, human lung carcinoma cells. Manuscript resubmitted with revisions to BMC cancer.

Geletu, M. Trotman-Grant, A and Raptis, L. (2012). Mind the gap; regulation of gap junctional, intercellular communication by the Src oncogene and its effectors. ***Anticancer Research***, in press.

Manuscripts submitted

Geletu, M , Arulanandam, R., Chevalier, S., Feracci, H. and Raptis, L. Classical cadherins control cytokine secretion and survival. Manuscript in the last stages of preparation for submission to *Oncogene.*

Geletu, M, Greer, S, and Raptis, L. Differential effects of polyoma virus middle tumor antigen mutants upon gap junctional, intercellular communication. In preparation for submission to the *Journal of Virology.*

Abstracts and scientific conference presentations

Invited Talk:

Queen's University Department of Biomedical and Molecular Sciences April, 2012.
Title: Little caves on the membrane: caveolin-1 and cell signaling

Abstract with oral presentation

Mulu Geletu, Reva Mohan, Rozanne Arulanandam, Adina Vultur, and Leda Raptis.2012 Reciprocal regulation of caveolin-1 and Stat3 in normal fibroblasts and breast carcinoma lines. Annual resident and

postdoctoral fellow research day February 28, Department of Pathology, Queen's University, Kingston, Ontario, Canada.

Abstracts with poster presentations

Mulu Geletu Leda Raptis, , Rozanne Arulanandam. Cadherin-11 function is required for full neoplastic transformation by v-Src. 103th annual meeting of the American association for Cancer research, March 31-April 4, 2012, Chicago, Illinois

Mulu Geletu, Reva Mohan, Rozanne Arulanandam, Adina Vultur, and Leda Raptis. 2012 Reciprocal regulation of caveolin-1 and Stat3 in normal fibroblasts and breast carcinoma lines. 103th annual meeting of the American association for Cancer research, March 31-April 4, 2012, Chicago, Illinois

Mulu Geletu, Rozanne Arulanandam, Carmeline D'Abreo, Leda Raptis. 2011 Cadherin-11 function is required for full neoplastic transformation by v-Src. The inaugural Canadian Cancer Research Conference, November 27-30, 2011 in Toronto, Ontario.

Mulu Geletu, Samantha Greer, and Leda Raptis. 2011 Activated phosphatidylinositol-3 kinase: An oncogene that increases gap junctional communication. The inaugural Canadian Cancer Research Conference, November 27-30, 2011 in Toronto, Ontario.

Conclusions

Our finding of Stat3 activation by cadherin engagement has exposed Stat3 as a central determinant of the balance between cell proliferation and apoptosis. Cadherin -11 knockdown experiments demonstrated that cadherin11 is required for Rac and Stat3 activation at high cell densities and this promotes survival, proliferation and migration. We also further demonstrate for the first time that the mesenchymal, class I cadherin, N-cadherin whose activation correlates with metastasis, can also trigger a dramatic surge in Stat3 in a similar manner. The cadherin/Stat3 axis is required for survival of tumor cells, which may be prone to apoptosis due to their higher E2F levels. Taken together, these data suggest that interference with cadherin-11 or N-cadherin function could induce apoptosis through Stat3 inhibition in metastatic cells specifically, a fact which could have important therapeutic implications.

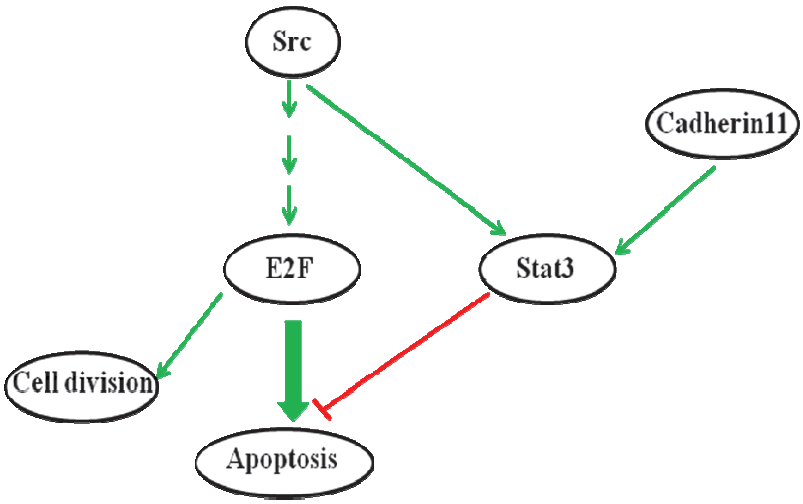

Figure 1

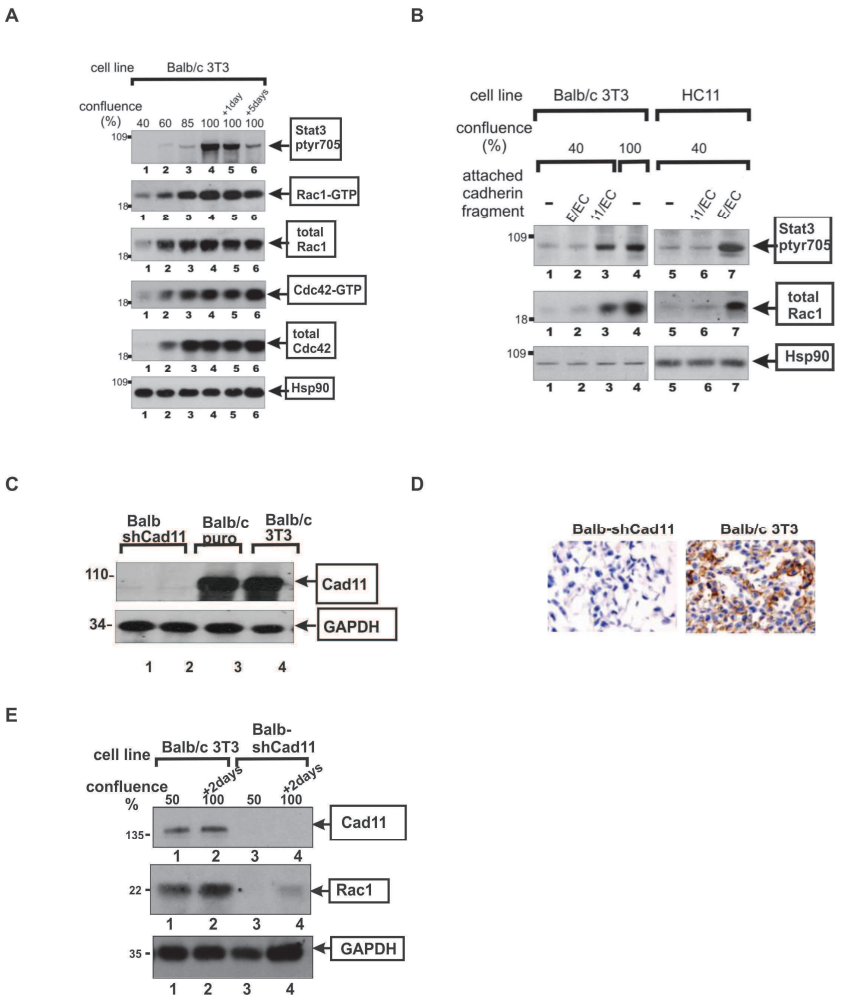

Figure 1

A. Cell density increases the activity as well as protein levels of Rac1 and Cdc42 in Balb/c 3T3 cells.

Balb/c 3T3 cells were grown to different densities, up to 5 days post-confluence, as indicated. Detergent cell lysates were probed for Stat3-ptyr705, active Rac-GTP, total Rac, active Cdc42, total Cdc42 or Hsp90 as a loading control, as indicated. Numbers at the left refer to molecular weight markers.

B. Cadherin-11 engagement is sufficient to increase Rac1 protein levels.

Balb/c3T3 cells were grown in plastic petris coated with 1,000 μg/ml of the 11/EC fragment or the E-cadherin-derived, E/EC fragment. 48 hours later, detergent cell extracts were probed for Stat3-ptyr705, Rac or Hsp90 as a loading control, as indicated. Numbers at the left refer to molecular weight markers.

C. Cadherin-11 knockdown causes a dramatic decrease in Rac1 levels. Balb-shCad11 cells were grown to different densities and cell extracts probed for cadherin-11, or GAPDH as a loading control.

D. Balb/c3T3 or shCad11-Balb/c3T3 cells were stained for cadherin-11. Notice the absence of staining in shCad11-Balb/c3T3 cells.

E. Extracts from control Balb/c3T3 cells (lanes 1-2) or shCad11-Balb/c3T3 cells (lanes 3-4) were probed for cad11, Rac1 or GAPDH as a loading control. Note the dramatic reduction in Rac1 levels in sh-Cad11-expressing clones.

Figure 2

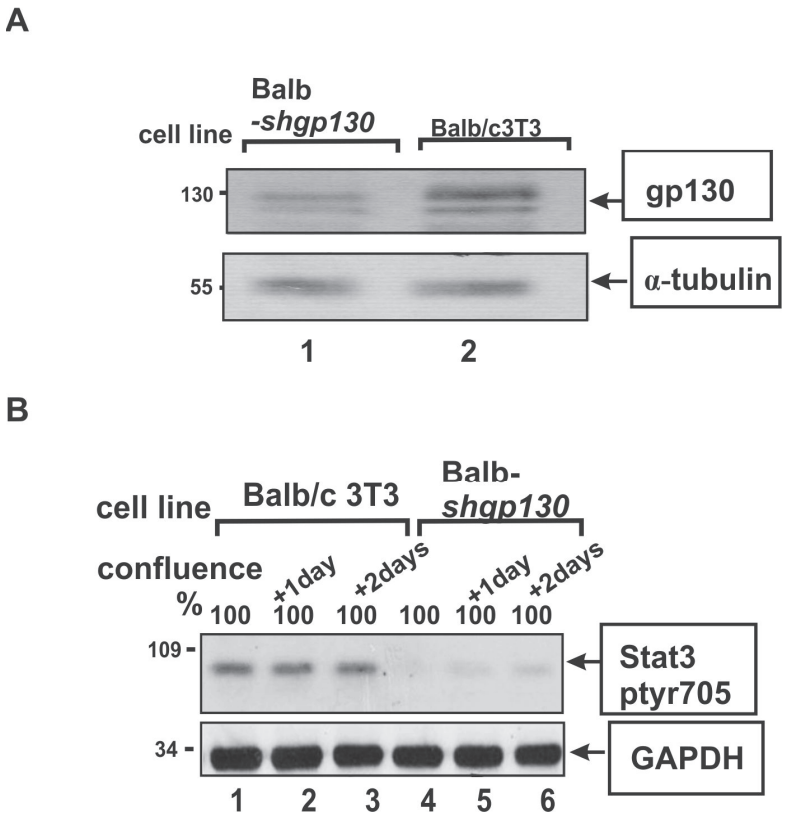

Figure 2

Gp130 knockdown causes a dramatic reduction in Stat3-ptyr705

A: Extracts from Balb/c3T3 cells before (lane 1) or after (lane 2) gp130 knockdown were probed for gp130 or tubulin as a loading control.

B: Extracts from Balb/c3T3 cells before (lanes 1-3) or after (lanes 4-6) gp130 knockdown were probed for Stat3-ptyr705 or GAPDH as a loading control.

Figure 3

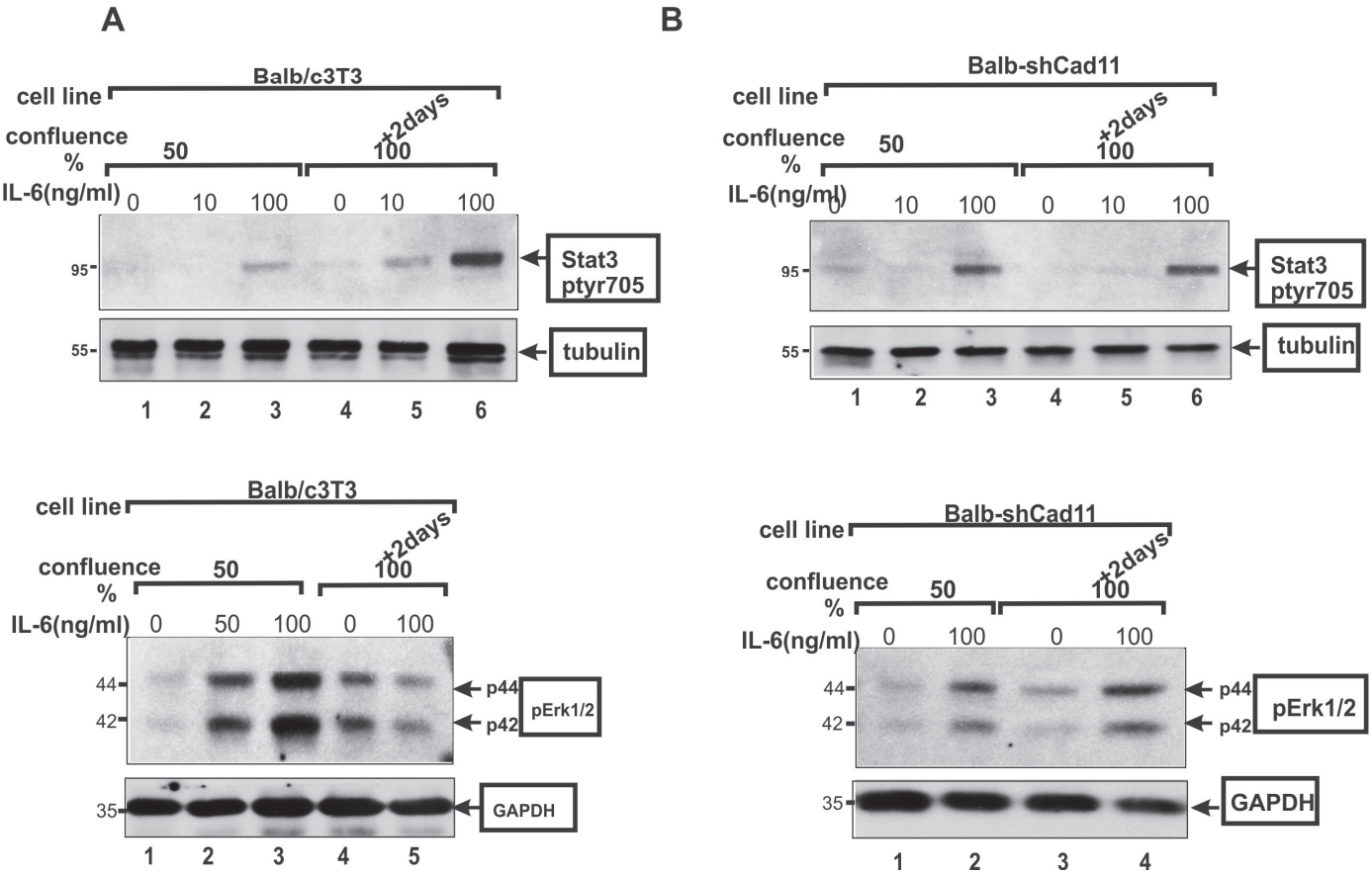

Figure 3

A: IL6 activates Stat3, but not Erk, at high densities in Balb/c3T3 cells. IL6 was added at 0, 10, 50 or 100 ng/mL for 15 min to Balb/c3T3 cells grown to 50% (lanes 1-3 or 2 days lanes 4-6) post confluence as indicated and cell extracts probed for Stat3-ptyr705 (upper panel) or Hsp90 as a loading control. Note the absence of Erk activation at high densities (lower panel, lanes 4 and 5).

B: IL6 activates Stat3 and Erk in the absence of cadherin-11. Same as above, cadherin11-deficient, Balb-shCad11 cells. Note the Erk activation at high densities (lower panel lanes 3 and 4).

Figure 4

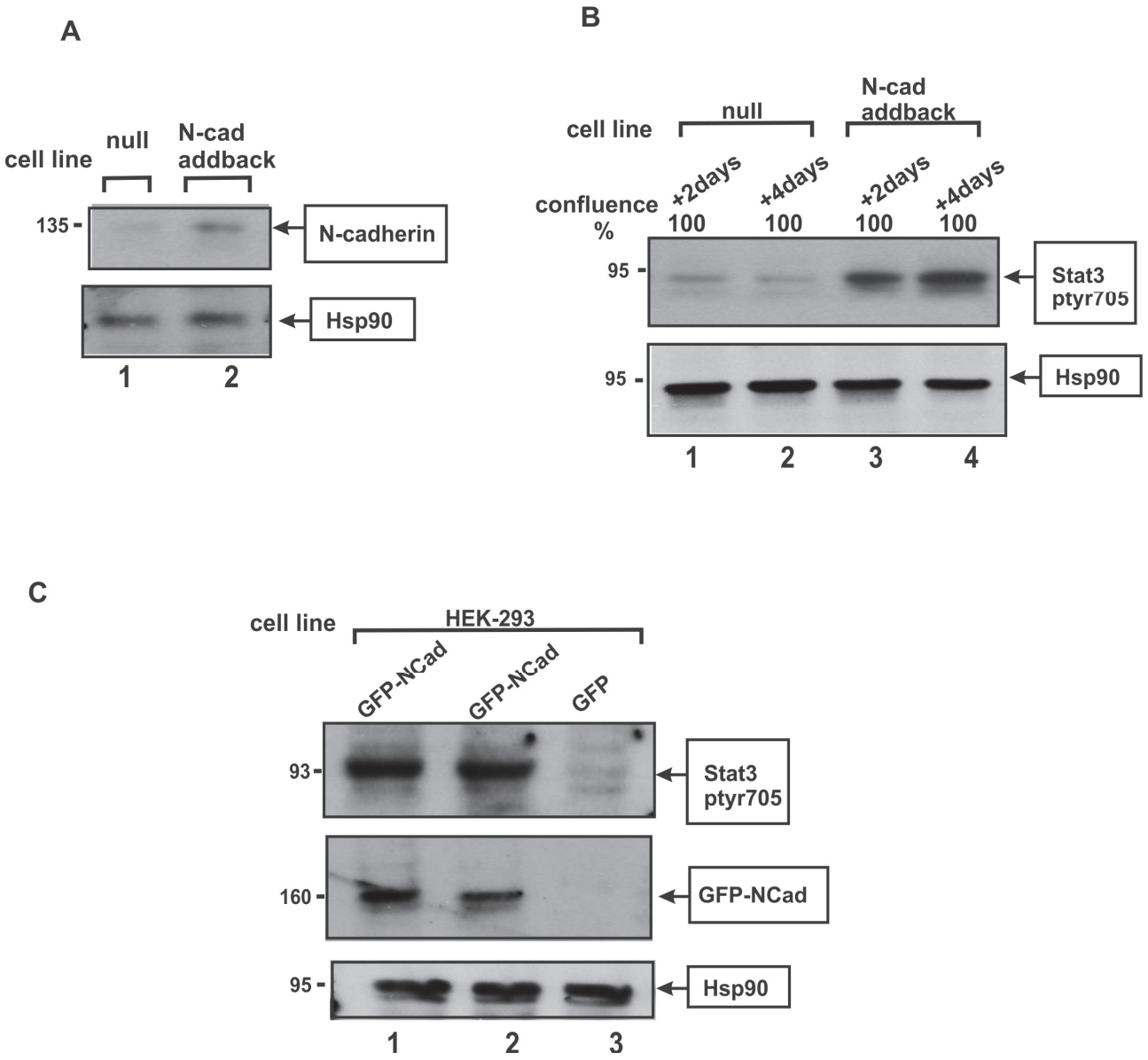

Figure 4

A: ES cells where E-cadherin was genetically ablated (null cells, lane 1) or null cells where N-cadherin was re-expressed (lane 2) were probed for N-cadherin or Hsp90 as a loading control.

B: Null cells (lanes 1-2) or N-cadherin addback cells (lanes 3-4) were probed for Stat3-ptyr705 or Hsp90 as a loading control.
C: HEK-293 cells were transfected with a GFP-Ncadherin plasmid using 5μg (lane 1) or 10μg (lane 2) per 6 cm plate or GFP alone (lane 3), and cell extracts probed 70 hrs later for Stat3-ptyr705, N-cadherin, or Hsp90 as a loading control.

Figure 5

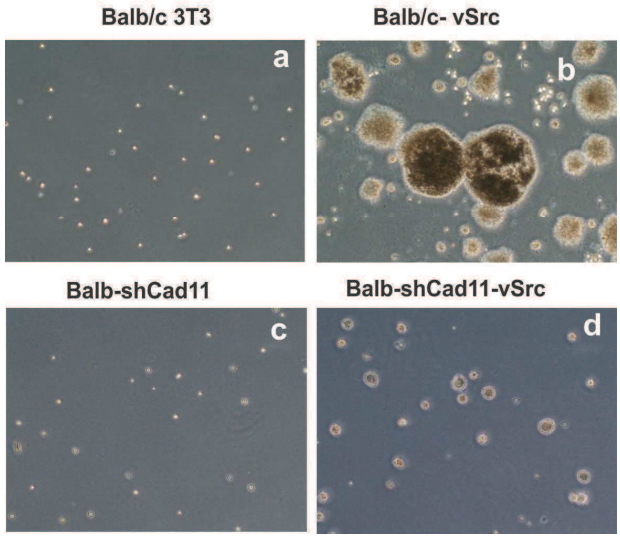

Figure 5

Cadherin-11 is needed for Src-transformation. Anchorage-independent growth:
(a) Balb/c3T3, (b) Balb/c3T3-vSrc, (c) Balb/c3T3-shcad11 and(d) Balb/c3T3-shcad11-vSrc, cells were suspended in soft agarose. Twenty days later cell were photographed under phase contrast illumination. Magnification: X40.

Figure 6

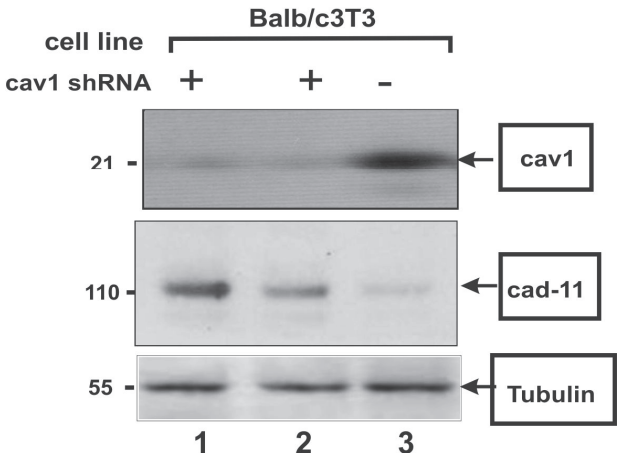

Figure 6

Cav1 downregulation increases Cadherin-11. Balb/c3T3 mouse fibroblasts were infected with the supernatant of the psi-2 cav1 shRNA packaging line, which produces the retrovirus carrying the *cav1 shRNA* gene and stably selected using puromycin as a selection marker. Infected cells (lanes 1-2) and the parental line (lane 3) were grown and lysates probed for total cav1, cadherin 11 or tubulin as a loading control.

Figure 7

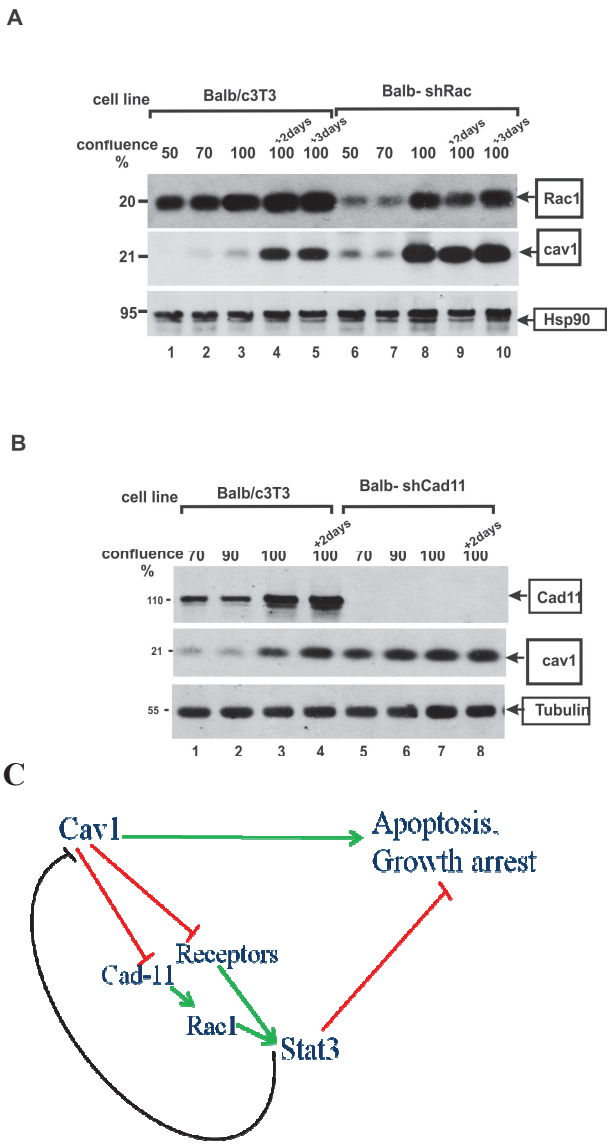

Figure 7:

Cadherin 11 and Rac1 downregulation increases cav1 level

A: Rac1 knockdown causes a dramatic increases cav1 levels. Extracts from control Balb/c3T3 cells (lanes 1-5) or sh-Cad11-expressing, cells (lanes 6-10) were probed for rac1, cav1or HSP90 as a loading control. Note the dramatic increase cav1 levels in sh-Rac1 expressing cells.

B: Cadherin-11 knockdown causes a dramatic increases cav1 levels. Balb/c3T3 (lanes 1-4) and Balb-shCad11 cells (lanes 5-8) were grown to different densities and cell extracts probed for cadherin-11, cav1 or tubulin as a loading control. Note the dramatic increase in cav1 levels in sh-Cad11-expressing cells.

C: Proposed model of cav1 action: Cav1 blocks cadherin-11 function, in addition to downregulating receptor action. Cadherin-11, through Rac1 was shown to activate Stat3, which is a potent survival signal. At the same time, Stat3 blocks cav1 expression.

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Appendices: attached

Stat3 is a positive regulator of gap junctional intercellular communication in cultured, human lung carcinoma cells

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Running Title: gap junctions, Src and Stat3 in lung cancer lines

Abstract

Background: Neoplastic transformation of cultured cells by a number of oncogenes such as *src* suppresses gap junctional, intercellular communication (GJIC); however, the role of Src and its effector Signal transducer and activator of transcription-3 (Stat3) upon GJIC in non small cell lung cancer (NSCLC) has not been defined. Immunohistochemical analysis revealed high Src activity in NSCLC biopsy samples compared to normal tissues. Here we explored the potential effect of Src and Stat3 upon GJIC, by assessing the levels of tyr418-phosphorylated Src and tyr705-phosphorylated Stat3, respectively, in a panel of NSCLC cell lines.

Methods: Gap junctional communication was examined by electroporating the fluorescent dye Lucifer yellow into cells grown on a transparent electrode, followed by observation of the migration of the dye to the adjacent, non-electroporated cells under fluorescence illumination.

Results: An inverse relationship between Src activity levels and GJIC was noted; in five lines with high Src activity GJIC was absent, while two lines with extensive GJIC (QU-DB and SK-LuCi6) had low Src levels, similar to a non-transformed, immortalised lung epithelial cell line. Interestingly, examination of the mechanism indicated that Stat3 inhibition in any of the NSCLC lines expressing high endogenous Src activity levels, or in cells where Src was exogenously transduced, did not restore GJIC. On the contrary, Stat3 downregulation in immortalised lung epithelial cells or in the NSCLC lines displaying extensive GJIC actually suppressed junctional permeability.

Conclusions: Our findings demonstrate that although Stat3 is generally growth promoting and in an activated form it can act as an oncogene, it is actually ***required*** for gap junctional communication both in nontransformed lung epithelial cells and in certain lung cancer lines that retain extensive GJIC.

Key words: Stat3, electroporation, Indium-Tin oxide, gap junctions, Src, cell to cell adhesion, lung cancer

Background

Gap junctions are plasma membrane channels that provide a path of direct communication between the interiors of neighboring cells and are formed by the connexin (Cx) family of proteins. An increase in cell proliferation correlates with a reduction in gap junctional, intercellular communication (GJIC [1]). In fact, a number of oncogene products such as v-Src [2], the polyoma virus middle Tumor antigen, an oncogene which acts by activating Src family kinases (mT [3,4]), the chaperone Hsp90N [5], vRas [6,7] and others have been shown to interrupt junctional communication.

Extensive evidence has indicated that expression of the Src tyrosine kinase leads to gap junction closure, through phosphorylation of the ubiquitous connexin, Cx43. Src exerts its effect either through direct tyrosine phosphorylation of Cx43, or indirectly, through activation of the serine/threonine, Erk1/2 or protein kinase C family kinases [8]. Examination of levels of tyr-418 phosphorylated, ie activated Src in a number of Non Small Cell Lung Cancer (NSCLC) biopsies previously showed the presence of higher Src activity than the surrounding, non-tumor lung tissue [9,10]. However, Src's contribution to GJIC suppression in NSCLC lines and primary cells which may express other oncogenes in addition to Src, or different levels of Src effectors, remains to be determined.

The Signal Transducer and Activator of transcription-3 (Stat3), an important Src downstream effector, is a cytoplasmic transcription factor. Following phosphorylation on tyr-705 by Src, as well as by growth factor or cytokine receptors such as the IL6 family, Stat3 normally dimerises through a reciprocal SH2 domain-phosphotyrosine interaction and translocates to the nucleus, where it induces the transcription of specific genes [11]. Examination of Stat3 levels in certain NSCLC lines demonstrated that Src is a major Stat3 activator in these cells, transducing signals from EGFR and IL6 that lead to apoptosis inhibition [12]. In another report [13] Src inhibition in certain NSCLC lines was found to actually increase Stat3-ptyr705. However, we and others previously demonstrated that cell-to-cell adhesion, as observed at confluence of cultured cells, causes a dramatic increase in Stat3 activity levels ([14-16] reviewed in [17]); for this reason, cell density must be taken into account in the examination of the effect of different factors upon Stat3 activity levels. In the present report this was achieved by measuring Stat3-ptyr705 phosphorylation and activity levels at a range of densities.

We previously assessed GJIC in a number of lung cancer lines [18]. In the present work GJIC was examined using an apparatus where cells were grown on a glass slide, half of which was coated with electrically conductive, optically transparent, indium-tin oxide. An electrode was placed on top of the cells and an electrical pulse, which opens transient pores on the plasma membrane, was applied in the presence of the fluorescent dye, Lucifer yellow. Although this technique is adequate for a number of lines, the turbulence generated as the electrode is removed can cause cell detachment, which makes GJIC examination problematic. Here, we revisited the question of GJIC levels in lung cancer lines using an improved technique, where the upper electrode is eliminated. This approach is valuable for the electroporation of tumor-derived lines especially at high densities, when cell adhesion to the substratum may be weak. Interestingly, the results revealed that cell density *per se* triggers a dramatic increase in both Cx43 levels and GJIC. Two NSCLC lines, QU-DB and SK-LuCi6 were found to have extensive GJIC, similar to control, nontransformed lung epithelial cells, while GJIC in five other lines was very low or undetectable. Investigation of the mechanism of gap junction closure revealed an inverse relation between Src activity levels and GJIC. Further studies led to the discovery that, unlike Ras inhibition in Src-transformed fibroblasts [19], Stat3 inhibition in NSCLC lines with high Src

activity does not restore GJIC. On the contrary, Stat3 inhibition in lines displaying extensive GJIC (QU-DB, SK-LuCi6) suppressed junctional permeability, indicating that Stat3 activity is actually ***required*** for the maintenance of gap junction function in these lung cancer lines.

Results

Cell density upregulates GJIC and connexin-43 protein levels

A number of reports showed that gap junction function is dependent upon cell to cell contact and the assembly of adherens junctions [20,21]. Since the opportunity for engagement of cadherins, key components of adherens junctions, is expected to increase with cell density, we examined the effect of cell density upon GJIC. To this effect, we took advantage of the nontransformed mouse lung epithelial type II line, E10 that has extensive GJIC, an even and flat morphology and good adhesion to the substratum even at high densities [22] (Fig. 1A). In addition, unlike nontransformed human lung lines such as NL-20 [23], E10 cells can be grown in the absence of growth factors that could affect GJIC. Cells were plated in electroporation chambers and when 90% confluent or at 3 days post-confluence Lucifer yellow was electroporated and the movement of the dye through gap junctions observed under fluorescence and phase contrast illumination (see Methods). The results are presented as the average number of cells where dye transfered, per cell loaded with the dye by electroporation (GJIC). As shown in Fig. 1B, ***a-c***, although cells at 90% confluence did display some gap junction transfer (GJIC ~1.5), GJIC increased to ~6 at 3 days post-confluence (Fig. 1B, ***d-f***), indicating that cell density causes a dramatic increase in GJIC.

We next examined the levels of Cx43, a widely expressed gap junction protein, at different cell densities. Cells were plated in plastic petri dishes at a confluence of 80% and at different times up to 5 days post confluence (Fig.1A, lane 1 *vs* 5), total protein extracts were probed for Cx43 by Western blotting. As shown in Fig. 1C, cell density caused a dramatic increase in Cx43 levels, which plateaued at ~3 days post-confluence.

GJIC and connexin-43 in NSCLC lines and freshly explanted tumor cells

In light of the above findings, we examined GJIC levels at different densities up to 4 days post-confluence in a panel of human lung cancer lines [18]. Two NSCLC lines, QU-DB (Fig. 2A, ***a-c***) and SK-LuCi6 (Table I) displayed extensive GJIC at their peak density, while five NSCLC lines had very low GJIC (e.g. A549, Fig. 2B, ***a-c,*** and Table I). In addition, primary cells explanted and cultured from a moderately differentiated adenosquamous carcinoma (Fig. 3, ***a-b***), a poorly differentiated adenocarcinoma, and an adenocarcinoma (Table I) had no GJIC.

Examination of Cx43 levels showed that QU-DB cells had levels similar to E10, which increased dramatically with cell density, while Cx43 levels in A549 cells were almost undetectable, at any cell density (Fig. 2C). SK-LuCi6 cells had levels similar to QU-DB, while all other NSCLC lines examined had very low Cx43 at all densities tested (not shown). The above data taken together indicate that, besides nontransformed epithelial cells, cell density causes a dramatic increase in GJIC and Cx43 protein levels in two lung carcinoma lines which display extensive GJIC. Nevertheless, the majority of lung cancer lines examined (5/7) had very low or no detectable gap junctional communication, even at high cell densities (Table I).

Src activity and GJIC suppression in NSCLC lines

We next examined Src-tyr418 phosphorylation, as an indication of Src activity. As shown in Fig. 4 (A and C), A549 cells displayed high Src-ptyr418 levels, similar to the levels in E10 or SK-LuCi6 cells expressing activated Src by retroviral transduction (lines E10-***Src***, SK-LuCi6-***Src***, respectively, Fig. 4C, lanes 1 *vs* 3 and Table II, B), while Src-ptyr418 levels in QU-DB cells were low (Fig. 4A, lanes 5-8), similar to E10 (Fig. 4B, lanes 5 and 6). Lines CALU-1, SW-900, CALU-6 and SK-Lu1 had Src-ptyr418 levels comparable to SK-LuCi6-***Src*** (Fig. 4B, lanes 1-3 *vs* 7 and Table II,B), while SK-LuCi6 had low levels, similar to QU-DB (Fig. 4B, lanes 4-5). Examination of gap junctional communication revealed that five lines with high Src-ptyr418 (A549, CALU-1, SW-900, CALU-6, LuCi-1) had very low or no detectable GJIC (Fig. 2B, ***a-c*** and Table II, B). In addition, Src expression in SK-LuCi6 or E10 cells eliminated junctional permeability (E10-***Src*** and SK-LuCi6-***Src***, Table II, B), in agreement with the known Src effect of GJIC suppression. Conversely, the two lines with low Src-ptyr418 levels (QU-DB and SK-LuCi6), had high GJIC, especially at high densities (Fig. 2A and Table II, A). Primary cells from the three tumor specimens were found to have higher Src activity than the E10, consistent with previous results from biopsy tissues (Fig. 3, bottom panel). Taken together, these data point to an inverse relationship between Src activity levels and GJIC in these NSCLC lines.

Stat3 is a positive regulator of GJIC in NSCLC lines

Stat3 is a prominent effector of the non-receptor tyrosine kinase Src [24]. In addition, Stat3 can be activated by cytokine and membrane tyrosine kinase receptors, which can act in a Src-independent manner [11]. Therefore, to assess the specific contribution of Src to Stat3 activation in the lung cancer lines, we at first examined the correlation between Src-ptyr418 and Stat3-ptyr705 levels. As shown before for a number of cell types (reviewed in [17]), high cell density was found to cause an increase in Stat3-ptyr705 levels in all lines (e.g. Fig. 4A, lanes 1-4 and 5-8) and its localisation to the nucleus, therefore Stat3-ptyr705 levels were assessed at a confluence of 50% for this experiment (see Methods). The results showed elevated Stat3-ptyr705 levels in the five lines with high Src-ptyr418 at all cell densities, comparable to SK-LuCi6-***Src*** cells (e.g. A549 *vs* QU-DB, Fig. 4A, lanes 1-4 *vs* 5-8 and Fig. 4B and Table II,B). At the same time, QU-DB and SK-LuCi6 cells had low levels of both Src-ptyr418 and Stat3-ptyr705 (Fig. 4B). The above data point to a correlation between Src and Stat3 activity levels in the NSCLC lines. We next examined the effect of Src inhibition upon Stat3-ptyr705 in the lines found to have high Src-ptyr418. The results showed that Src inhibition with Dasatinib caused a dramatic reduction in Stat3-ptyr705 (e.g. line A549, Fig. 4C, and Additional data, Table Add-I), indicating that Src may, in fact, be an important Stat3 activator in these cells.

We next examined the effect of Stat3 inhibition upon GJIC in the 5 lines with high Src activity. As shown in Fig. 2 (E and F) treatment with the Stat3 inhibitor, CPA7 for 24 hrs [25], or knockdown with a Stat3-specific, shRNA, essentially eliminated Stat3, tyr705 phosphorylation in A549 cells. Unexpectedly however, CPA7 treatment (Fig. 2B, ***d-f***), or Stat3 knockdown (Fig. 2B, ***g-i***) did not increase junctional permeability in A549 cells. Similar results were obtained with SK-Lu1, CALU-1, SW-900 and CALU-6 lines (Table II,B). The above data taken together indicate that the high Stat3 activity, which could be, at least in part, due to high Src activity in these lines, cannot be responsible for the lack of junctional communication in the lung carcinoma lines examined.

Since the lung cancer lines might express other oncogenes besides Src, we examined the role of Stat3 in the Src-mediated GJIC suppression specifically, using the Src-transduced, SK-LuCi6-***Src*** line. As expected, Src expression disrupted gap junctional permeability.

Interestingly, subsequent Stat3 inhibition with CPA7 or shRNA did not restore GJIC (Table II). Taken together, the above findings indicate that Stat3 cannot be part of a pathway leading to Src-induced, gap junction closure in SK-LuCi6-***Src*** cells.

We then examined the possibility that Stat3 might play a ***positive*** role in the maintenance of gap junctional permeability, by assessing the effect of Stat3 inhibition upon GJIC levels in QU-DB cells which have low Src activity and extensive GJIC. Unexpectedly, as shown in Fig. 2A (***d-f***), Stat3 downregulation through CPA7 treatment essentially ***abolished*** GJIC in QU-DB cells. Reduction of Stat3 levels through infection with the sh-Stat3 lentivirus vector gave similar results (Fig. 2A, ***g-i***). Similarly, Stat3 downregulation in SK-LuCi6 or E10 cells caused a dramatic ***de***crease in GJIC (Table II,A). In addition, expression of the constitutive form of Stat3, Stat3C {Bromberg 1999}, increased gap junctional communication (Table I).

Examination of Cx43 levels following Stat3 inhibition revealed a dramatic reduction following sh-Stat3 expression (Fig. 2D), indicating that Stat3 is required for the maintenance of Cx43 protein levels. Examination of apoptosis revealed that CPA7 treatment did increase the levels of the apoptotic protease, caspase-3. Caspase-3 levels were higher at the higher cell densities where GJIC is studied, which hints at a link between GJIC reduction and apoptosis induced by Stat3 inhibition. Taken together, our data reveal that, rather than increasing junctional permeability as might have been expected based on the well documented ability of Stat3 to act as a Src effector, Stat3 inhibition eliminates GJIC, indicating that Stat3 activity is actually ***required*** for gap junction function in two cultured lung carcinoma lines which display extensive GJIC.

Discussion

Extensive data from our group and others demonstrated that oncogenes such as mT, Src or Ras can suppress gap junctional, intercellular communication [3,6]. Moreover, it was shown that lower levels of these gene products were sufficient to eliminate gap junction function than the levels necessary for full transformation [4,26], indicating that a decrease in GJIC may be an early event in neoplastic conversion. In this communication we used an improved procedure to examine GJIC in lung cancer lines as well as in primary lung tumor cells. All cell lines had been established from NSCLC tumors which were known to be metastatic [18], except QU-DB, which was derived from a patient that was a long term survivor [27]. Our results reveal that GJIC was low in the majority of cases, except in the QU-DB and SK-LuCi6 lines. Assuming that the establishment process did not bring about an ***increase*** in GJIC, the existence of extensive GJIC in line SK-LuCi6 which was established from a rapidly metastatic tumor [28] indicates that intercellular communication does not necessarily inhibit metastasis; other factors may supercede potential growth inhibitory effects of intercellular communication and may be responsible for tumor growth and metastasis.

We next examined the mechanism of GJIC suppression by assessing the role of Src and its effector Stat3. Our results revealed an inverse relationship between Src-tyr418 phosphorylation levels and GJIC in a number of lines. Since Src is known to suppress gap junctional communication in cultured cells such as rodent fibroblasts and epithelial cells, it is tempting to speculate that Src may be responsible, at least in part, for gap junction closure in these lines. However, repeated attempts to reinstate GJIC by reducing Src activity levels through treatment with the Src kinase family-selective, pharmacological inhibitors Dasatinib, PD180970 or SU6656, or infection with Adenoviral vectors expressing a Src dominant-negative mutant or c-Src kinase [15] in A549 cells which have high Src-ptyr418 were unsuccessful (not shown). Possibly other oncoproteins besides Src, or other factors may be important contributors to GJIC

suppression in these lines. Alternatively, since low levels of activated Src were previously shown to be sufficient for GJIC suppression in mouse fibroblasts [3,4], the possibility that the residual Src activity in treated cells might be sufficient to interrupt gap junctional communication cannot be excluded.

We also examined GJIC in freshly explanted, primary cells from 3 NSCLC specimens. Since the senescence process can reduce GJIC [29], cells were plated in electroporation chambers immediately after surgery at densities of ~80%, so that they would reach confluence within 1-2 days, and GJIC examined every day for up to 10 days. No gap junctional communication was ever detected in any of the preparations, although fibroblasts from the same tissue had extensive GJIC (Fig. 3, ***c-d***). Src-418 levels were relatively high in cells from all three tumor specimens, indicating that Src may have played a role in GJIC suppression. However, the possibility that the initiation of the senescence process even a day after surgery may have affected GJIC cannot be excluded.

Stat3 does not transmit Src signals to gap junction closure

Several signal transducers besides Stat3 are known to be downstream effectors of the Src kinase such as Ras/Raf/Erk, PI3k/Akt, the Crk-associated substrate (Cas) and others [30]. Constitutively active Ras is neoplastically transforming and can suppress GJIC [6,26]. Examination of the mechanism of Src-mediated, GJIC suppression previously indicated that inhibition of Ras in Src-transformed, rat fibroblasts reinstated gap junctional communication [19]. Conversely, mT expression in Ras-deficient cells did not suppress GJIC [31]. These data taken together underline the importance of the Ras pathway in GJIC reduction by activated Src. It was also shown later that Cas is required for the Src-induced, reduction in gap junctional communication [32]. In sharp contrast, our present data with Src-transduced, SK-LuCi6-***Src*** cells demonstrate that Stat3 inhibition does not restore GJIC, indicating that a role of Stat3 in the Src-induced, GJIC suppression in these cells is unlikely, despite the fact that constitutively active Stat3 can act as an oncogene and transform established lines [33].

Stat3 plays a *positive* role in gap junctional communication

The fact that cell density upregulates Stat3 concomitant with an increase in both Cx43 and GJIC prompted us to explore a potential positive role of Stat3 upon GJIC. Interestingly, Stat3 inhibition in two NSCLC lines which exhibit extensive junctional communication (QU-DB, SK-LuCi6) abolished GJIC, indicating that Stat3 does in fact play a positive role in the maintenance of gap junction function. This conclusion is in agreement with a previous report indicating that Stat3 inhibition eliminated GJIC in nontransformed rat liver epithelial cells as well [34].

Our data also show high Stat3 activity in SK-LuCi6-***Src*** cells, as well as five NSCLC lines, which could be at least in part due to the high Src activity (Fig.4D). However, despite the fact that Stat3 apparently promotes GJIC in nontransformed cells, the Src-mediated, Stat3 activation does not lead to an increase in gap junctional permeability. This could be due to the concomitant Cx43 phosphorylation by Src, either directly on tyrosine or indirectly on ser/thr, followed by degradation [8,35], so that the net effect of Src expression is gap junction closure.

Results from a number of labs demonstrated that Stat3 activates a number of anti-apoptotic genes, such as BcL-xL and Akt1 [11]. Global induction of apoptosis was shown to lead to a loss of cell coupling, probably due to caspase-3-mediated degradation of Cx43, in primary bovine lens epithelial and mouse NIH3T3 fibroblasts [36]. Interestingly, we previously

demonstrated that Stat3 inhibition in cells transformed by Src or the Large Tumor antigen of Simian Virus 40 leads to apoptosis [15,37], possibly due to activation of the transcription factor E2F family, potent apoptosis inducers, by these oncogenes. Therefore, apoptosis induced by Stat3 downregulation in cells with high Src may have accentuated gap junction closure.

We previously demonstrated that while Stat3 inhibition in normal mouse fibroblasts causes a growth retardation, at high densities, such as needed for optimal gap junction formation, Stat3 inhibition leads to apoptosis [38]. Therefore, apoptosis induction through a reduction in Stat3 levels or activity could explain the dramatic reduction in Cx43 and GJIC upon Stat3 pharmacological or genetic inhibition.

Conclusions: Our results demonstrate that Stat3 is not transmitting Src signals leading to gap junction closure. In the contrary, although Stat3 is generally growth promoting and in an activated form it can act as an oncogene, we show for the first time that Stat3 is actually ***required*** for gap junctional communication both in normal epithelial cells and in certain tumor cell lines that retain GJIC. This novel role of Stat3 in gap junction function may be an important regulatory step in progression of tumours that exploit such a pathway.

Methods

Examination of gap junctional communication

To examine gap junctional communication by *in situ* electroporation, it is important to be able to reliably distinguish cells that were loaded with Lucifer yellow directly by electroporation, from cells that received the dye from neighbouring cells by diffusion through gap junctions. This was achieved using a slide where a 3 mm-wide strip of ITO had been removed by etching with acids, leaving two co-planar electrodes, supported by the same glass slide substrate (Fig. 5) [39].

In a further improvement (Fig. 6), the coating was removed from the glass surface in ~20 μm wide lines, to define electrode and non-conducting regions. Etching was done using a laser beam, so that the nonconductive glass underneath is exposed. It was important to ensure that only the 800Å coating was removed, without affecting the glass, so that cell growth would be unaffected across the line. This was achieved with a UV laser operating at a 355 nm wavelength using approximately 1 Watt of output power with 60% of the energy delivered to the surface of the glass. The beam was manipulated by mirrors on a pair of galvanometers to produce the desired pattern.

To form the two electrodes, the coating was removed in a straight line in the middle (**2**). A dam of nonconductive plastic (**3)** was bonded onto this line, to divert the current upwards, thus creating a sharp transition in electric field intensity between electroporated and non-electroporated sections. To provide areas where the cells are not electroporated, the ITO was also removed in two parallel lines [(**4) and** (**4*a***)]. A plastic chamber was bonded onto the slide, to form a container for the cells and electroporation solutions (**5)**. Current flows inwards from each contact point (**6** and **6*a***), via a conductive highway under the well (**5)** electroporating cells in area (***d***) then over the barrier [(**8)**, arrowheads] to the other side, in area (***a***). In this configuration, cells which acquired LY by electroporation [growing in (***a***) and (***d***)] and cells into which LY traveled through gap junctions [(***b***) and (***c***)] both grow on ITO, separated only by a laser-etched line of ~20 μm. Extensive experimentation showed that in this setup the electroporation intensity is uniform across the electroporated area (see Fig. 1B and Fig. 2).

Cells were plated in the chamber and when they reached the appropriate density (90% confluence, to 5 days post-confluence), the growth medium was replaced with Calcium-free DMEM supplemented with 5 mg/ml Lucifer yellow (**7**). The slide/chamber was placed into a holder where electrical contacts were established and a set of electrical pulses delivered to the cells. Extensive experimentation indicated that 10 pulse pairs, each pulse of 18 Volts peak value, 100 μs length and spaced 0.5 seconds apart, with one of each pair having a polarity opposite to that of its partner gave optimal results. Following a 5 min incubation at 37^{o}C, the unincorporated dye was washed away with Calcium-free DMEM supplemented with 10% dialysed fetal calf serum and cells observed and photographed under fluorescence and phase contrast illumination.

Cell lines, culture techniques and Stat3 activity measurement

All cells were grown in DMEM with 10% fetal calf serum. Extra care was taken to ensure that cell seeding was uniform, by passing cells at subconfluence, when cell to cell adhesion was low. Confluence was estimated visually and quantitated by imaging analysis of live cells under phase contrast [14]. To ensure that the growth medium was not depleted of nutrients, it was changed every day.

Cells were cultured from surgically explanted tumors as previously described [18].

Stat3 transcriptional activity was measured as described, by transient transfection of the p*Luc*TKS3 construct [24]. As a control, cells were co-transfected with the reporter pRLSRE, which contains two copies of the serum response element (SRE) of the c-fos promoter, subcloned into the *Renilla* luciferase reporter, pRL-null (Promega) [15]. Following transfection, cells were plated to different densities and luciferase activity determined.

Inhibitors Stat3 was inactivated using two approaches: **(1).** Treatment with 50 μM CPA7 [$PtCl_3(NO_2)(NH_3)_2$] [25] for 24 hrs **(2)**. Expression of shRNA, delivered with a lentivirus vector as described [34]. Jak inhibitor-1 was from EMD Biosciences (5 μM).

Src was inactivated using 3 pharmacological inhibitors: Dasatinib (1 μM, up to 72h), PD180970 (0.2 μM with redosing every 12h for a total of 24h), or SU6656 (5μM for 24h) [15].

Western blotting was conducted on proteins extracted from cell pellets [40], using antibodies to Cx43 (Cell Signalling, #3512, used at 1:500), Stat3-ptyr705 (Cell Signalling, #9131, 1:1,000), GAPDH (BD Transduction, #14C10, 1:5,000), Src-ptyr418 (Invitrogen, #44-660G , 1:1,000) or total Src (rabbit monoclonal 36D10, Cell Signalling, #2109, 1:1,000), followed by secondary antibodies and ECL reagents (Biosource). Alpha-Tubulin (Cell Signalling #2125, 1:5,000), or Hsp90 (Assay designs, #SPA-830, 1:5,000) served as loading controls.

Competing interests: The corresponding author received a royalty from PARTEQ, the intellectual property arm of Queen's University, for a patent where she is a co-inventor of the apparatus used in this work. PARTEQ had no involvement whatsoever in the content of this paper.

Authors' contributions: MG did the bulk of the benchwork. SG and RA conducted some of the initial experiments. Aaron Trotman-Grant did the Stat3C experiments. ET obtained the results on the primary lung carcinoma cells. LR conceived of the study, designed and coordinated it and drafted the manuscript. All authors read and approve of the manuscript.

Authors' information: MG is a postdoctoral fellow, funded by the US Army breast cancer program. RA and ET were graduate students. ATG is currently doing a project. LR is a professor at Queen's University, Kingston, Canada.

Abbreviations:
Cx43: connexin-43
GJIC: Gap junctional, intercellular communication
NSCLC: Non-small cell lung cancer

Acknowledgements

We would like to thank Dr. Mike Baird and Shallyn Littlefield for CPA7 synthesis, Dr Andrew Craig of Queen's University for the NL20 line and Kevin Firth, P.Eng., of Ask Sciences products, Kingston, Ontario for engineering design. We are grateful to Dr Elissavet Kardami (University of Manitoba, Canada) for a generous gift of connexin-43 antibody. The financial assistance of the Canadian Institutes of Health Research (CIHR), the Canadian Breast Cancer Foundation (Ontario Chapter), the Natural Sciences and Engineering Research Council of Canada (NSERC), the Canadian Breast Cancer Research Alliance, the Ontario Centers of Excellence, the Breast Cancer Action Kingston and the Clare Nelson bequest fund through grants to LR is gratefully acknowledged. RA was supported by a Canada Graduate Scholarships Doctoral award from CIHR, the Ontario Women's Health Scholars Award from the Ontario Council on Graduate Studies and a Queen's University Graduate Award. ET was the recipient of an OGSST award. SG was the recipient of an NSERC summer studentship. MG was supported by a postdoctoral fellowship from the US army Breast Cancer Program, the Ministry of Research and Innovation of the Province of Ontario and the Advisory Research Committee of Queen's University.

Table I

Effect of Stat3 downregulation upon GJIC

A. Cells with extensive junctional communication

Cell line	Treatment[α]	Src[β] (%)	Stat3[β] (%)		GJIC[γ]	
			50%	100+3d	90%	100+3d
E10	-	6±1	9±3	26±9	1.5±0.5	6.0±1
"	**DMSO**	6±1	9±3	30±8	-	6.0±1
"	**CPA7**	5±1	2±1.1	3±1	-	0.2±0.1
"	**sh-Stat3**	N/A	6±1.1	8±2	-	1.0±0.2
QU-DB	-	7±1	10±2	20±4	1±0.2	6.3±1
"	**DMSO**	7±1	10±2	22±3	-	6.3±1
"	**CPA7**	5±1	2±0.5	2±0.5	-	0.2±0.1
"	**sh-Stat3**	N/A	5±3	8±2	-	0.8±0.2
SK-LuCi6	-	5±1	8±2	21±4	1.8±0.2	6.5±1
"	**DMSO**	5±1	8±2	20±5	-	6.5±1
"	**CPA7**	5±1.2	2.8±1.25	3±1	-	0.3±0.1
"	**sh-Stat3**	N/A	6±2	4±0.5	-	1±0.2
"	**Jak inhib.1**	5.2±0.3	4.2±1.1	5±0.5	-	0.5±0.2
"	**Stat3C**	5.1±1	22±9	97±10	-	8±1

B. Cells expressing activated Src

Cell line	Treatment[α]	Src[β]	Stat3[β] (%)		GJIC[γ]	
			50%	100+3d	90%	100+3d
A549	-	95±11	93±12	320±32	0.1 ±0.1	0.3 ±0.1
"	**DMSO**	95±11	93±12	320±32	-	0.3 ±0.1
"	**CPA7**	93±10	8±1	12±2	-	0.1 ±0.1
"	**sh-Stat3**	N/A	12±3	11±4	-	0.1±0.1
E10-*Src*	**DMSO**	98±12	98±15	350±28	-	0.4 ±0.2
"	**CPA7**	95±11	5±1	15±4	-	0.1 ±0.1
"	**sh-Stat3**	N/A	9±3	20±3	-	0.1±0.1
SK-LuCi6-*Src*	**DMSO**	100±12	100±12	420±33	-	0.2±0.1
"	**CPA7**	98±10	3±1	9±1	-	0.1 ±0.1
"	**sh-Stat3**	N/A	11±3	17±3	-	0.3±0.1
SK-Lu-1	**DMSO**	85±5	90±11	311±23	-	1±0.2
"	**CPA7**	82±4	6±1	8±3	-	0
CALU-1	**DMSO**	96±9	100±10	290±12	-	0
"	**CPA7**	90±12	8±2	6±1	-	0
SW-900	**DMSO**	100±13	100±12	405±21	-	0
"	**CPA7**	96±11	12±1	11±2	-	0
CALU-6	**DMSO**	95±11	93±10	300±18	-	0
"	**CPA7**	93±10	10±1	15±5	-	0

[α]For Stat3 inhibition, cells were treated with 50 μM CPA7, or the DMSO carrier for 24 hrs, or infected with a lentivirus vector expressing a Stat3-specific, shRNA. For Stat3 upregulation, cells were infected with a retroviral vector containing Stat3C. Jak inhibitor-1 was used at 5μM.

[β] Stat3-tyr705 or Src-ptyr418 levels were measured by Western blotting. Numbers represent relative values obtained by quantitation analysis. Averages of at least three experiments ±SEM are shown. For Stat3, data from cells grown to 50% confluence or 3 days after confluence are presented [15], with the average of the values for DMSO-treated, Src-transduced, SK-LuCi6-***Src*** cells taken as 100%. The transcriptional activity values obtained paralleled the Stat3-705 phosphorylation levels indicated (Fig. 4, E and F, see Methods).

[γ]GJIC was assessed by *in situ* electroporation at the indicated confluences (see Methods, Fig. 6). Quantitation was achieved by dividing the number of cells into which the dye had transferred through gap junctions (denoted by dots, Fig. 1B and 2A), by the number of cells at the edge of the electroporated area (denoted by stars). Numbers are averages ±SEM of at least three experiments, where transfer from more than 200 cells was examined.

N/A: Not applicable

Table II
GJIC in primary lung carcinoma cells[α]

	Cells[β]	GJIC[α]
Adenosquamous carcinoma, moderately differentiated	**carcinoma cells**	0
	fibroblasts	5.8±1.2
Adenocarcinoma, poorly differentiated	**carcinoma cells**	0
Adenocarcinoma	**carcinoma cells**	0

[α]Immediately after surgery, cells were placed in culture and GJIC examined (see Methods, Fig. 5). After 8-10 weeks in culture, most of the tumor cells had died while the fibroblasts present in the initial suspension predominated. These cells did not express cytokeratins, contrary to tumor cells [18]. The fibroblasts shown were derived from the moderately differentiated adenosquamous carcinoma tumor above (Fig. 3, *c-d*). GJIC was examined as in Table I, at 3 days after confluence.

Figure legends

Figure 1

Cell density increases GJIC and Cx43 levels

A. Immortalised lung epithelial E10 cells were plated in 3 cm plastic petri dishes, grown to different densities and photographed under phase-contrast illumination. Magnification: 240x.

B. E10 cells were plated in electroporation chambers and subjected to a pulse in the presence of Lucifer yellow when 90% confluent (***a-c***) or 3 days after confluence (***d-f***) and photographed under phase-contrast (***a, d***), fluorescence (***b, e***) or combined (***c, f***) illumination (see Materials and Methods, Fig. 6). Arrows point to the position of the edge of the electroporated area. In ***a***, ***b***, ***d*** and ***e***, stars mark cells loaded with the dye at the edge of the electroporated area and dots mark cells into which the dye was transferred through gap junctions. Magnification: 240x.

C. E10 cells were seeded in plastic petri dishes and when they reached the indicated densities, detergent cell extracts were probed for Cx43 (top) or GAPDH (bottom) as a control.

Figure 2

A. Stat3 downregulation eliminates gap junctional permeability in human lung carcinoma QU-DB cells

QU-DB cells were plated in electroporation chambers and subjected to a pulse in the presence of Lucifer yellow, following treatment with the DMSO carrier alone (***a-c***), or CPA7 (***d-f***), or infection with the sh-Stat3 lentiviral vector (***g-i***) (see Materials and Methods, Fig. 6). After washing away the unincorporated dye, cells from the same field were photographed under fluorescence (***b, e, h***) or phase contrast (***a, d, g***) illumination. Cells at the edge of the conductive area which were loaded with LY through electroporation were marked with a star, and cells at the non-electroporated area which received LY through gap junctions were marked with a dot [4]. Arrows point to the edge of the electroporated area. ***c, f, i***: Overlay of phase-contrast and fluorescence. Magnification: 240x.

Note the extensive gap junctional communication in (***b***).

B. Stat3 downregulation does not increase gap junctional permeability in human lung carcinoma A549 cells

Same as above, A549 cells. Note the absence of GJIC, even after Stat3 downregulation (***e, h***).

C: Cell density causes a dramatic increase in Cx43 levels in QU-DB cells.

QU-DB (lanes 5-8) or A549 (lanes 1-4) or nontransformed E10 (lane 9) cells were grown to different densities as indicated and extracts probed for Cx43 or Hsp90 as a loading control. Note the absence of Cx43 in A549 cells and the increase in Cx43 with density in QUDB.

D: Stat3 knockdown reduces Cx43 levels

QU-DB cells infected with the lentiviral vector carrying the Stat3-specific shRNA (lane 2) or not infected (lane 1) were grown to 2 days post-confluence and lysates probed for Cx43 or Hsp90 as a loading control.

E: CPA7 or Stat3-knockdown with shRNA reduce Stat3-ptyr705 levels in A549 cells

A549 cells were grown to increasing densities and treated with the Stat3 inhibitor, CPA7 (lanes 6-8) or the DMSO carrier (lanes 1-5) for 24 hrs and cell extracts probed for Stat3-ptyr705 or tubulin as a loading control. Parallel cultures were infected with a vector expressing a Stat3-specific, shRNA [34], and cell extracts from stable lines produced were probed as above.

F: CPA7 or Stat3-knockdown with shRNA reduce Stat3 transcriptional activity in A549 cells

A549 cells were transfected with a plasmid expressing a firefly luciferase gene under control of a Stat3-responsive promotor (■) and a Stat3-independent promotor driving a *Renilla* luciferase gene (□) (see Materials and Methods). After transfection, cells were plated to different densities and treated with CPA7 or the DMSO carrier alone for 24 hrs, at which time firefly and *Renilla* luciferase activities were determined. Parallel cultures expressing the sh-Stat3 construct were transfected with the plasmids and firefly and *Renilla* luciferase activities determined.

Figure 3

Primary lung carcinoma cells display low gap junctional, intercellular communication
a and b: Cells cultured from a freshly explanted lung tumor specimen were grown in electroporation chambers and Lucifer yellow introduced with an electrical pulse (Fig. 5, [39]). Arrows point to the edge of the electroporated area. Note the absence of gap junctional communication. Magnification: 240x.

c and d: Following growth of the cells for 10 weeks, fibroblasts present in the original cell suspension predominated. They were plated in electroporation chambers and Lucifer yellow introduced with an electrical pulse. Note the extensive communication through gap junctions.

Lower panel

Extracts of cells cultured from a moderately differentiated adenosquamous carcinoma, a poorly differentiated adenocarcinoma, and adenocarcinoma, respectively (lanes 1-3), or E10 cells (lane 4), were probed for Src-ptyr418 or GAPDH as a loading control, as indicated.

Figure 4

A: A549 cells have high Src-ptyr418 levels

QU-DB (lanes 5-8) or A549 (lanes 1-4) cells were grown to different densities as indicated and extracts probed for Src-ptyr418, Stat3-ptyr705 or total Src. Note the low levels of Src-ptyr418 in QU-DB cells.

B: Src-ptyr418 and Stat3-ptyr705 in NSCLC lines.

The indicated cell lines were grown to 50% confluence and extracts probed for Src-ptyr418, Stat3-ptyr705, total Src or GAPDH as a loading control.

C: Dasatinib reduces Stat3-ptyr705 levels in A459 cells:

A549 or SK-LuCi6-***Src*** cells were grown to subconfluence and treated with the Src-selective inhibitor, Dasatinib or the DMSO carrier alone and cell extracts probed for Src-ptyr418, Stat3-ptyr705 or GAPDH as a loading control, as indicated.

Figures 5 and 6: The electroporation apparatus

Figure 5: Electroporation on two co-planar ITO electrodes, formed by removing the ITO coating by chemical etching

A: Top view. Cells were grown on an ITO-coated slide from which the coating was removed in a strip as shown. The two conductive sides (***a, f***), serving as electrodes, were connected to the positive and negative poles of the pulse generator (2) and (3). A nonconductive barrier (5) divides the strip of bare glass in half and separates the chamber into two sections.

B: Side view. The slide with the cells growing on the ITO coated and the bare glass regions is shown. When electroporation buffer is added to the chamber to a level above the height of the barrier (5) then an electrical path between the electrodes (***e*** and ***b***) is formed. Note that the ITO layer (1***a***) is shown with dramatically exaggerated thickness for clarity, although its actual thickness is much less than the thickness of the cells (from [39]).

Figure 6: Electroporation on two co-planar ITO electrodes, formed by removing the ITO coating by etching with Lasers

A: Top view. Cells are grown on a glass slide (**1**), coated with conductive and transparent indium-tin oxide [ITO, (**1*a***)]. The ITO coating is laser-etched in a straight line in the middle (**2**), essentially forming two electrodes. A dam of Teflon (**3**) is used to divert the current upwards, thus creating a sharp transition in electric field intensity. A plastic chamber is bonded onto the slide, to form a container for the cells and LY (**5**). To provide areas where the cells are not electroporated, the ITO was also removed in two parallel lines [(**4**), (**4a**)]. Current from a pulse generator flows inwards from each contact point (**6** and **6*a***) to area (***d***) then over the barrier [(**3**), arrowheads] to the other side, electroporating cells in area (***a***). For clarity, the front part of the chamber is removed.

B: Side view. The slide (**1**) with the cells growing on the ITO coated [(**1*a***), light green] and etched, bare glass regions is shown. When electroporation medium (**7**) is added to the chamber to a level above the height of the dam (**3**) then an electrical path (arrowheads) between the electrodes (**6**) and (**6*a***) and the cells (9) growing in this area is formed. Note that the size of the cells and the ITO layer (**1*a***) are shown exaggerated for clarity although the actual thickness of the ITO (800 Å) is much lower than the thickness of the cells.

Additional data

Table Add-I
Effect of Src downregulation upon Stat3-ptyr705

Cell line	Treatment[α]	Src[β] (%)	Stat3[β] (%)
A549	**DMSO**	95±11	93±12
"	**Dasatinib**	10±2	8±4
E10-*Src*	**DMSO**	98±12	98±15
"	**Dasatinib**	8±1	10±1
SK-LuCi6-*Src*	**DMSO**	100±12	100±12
"	**Dasatinib**	3±1	5±3
SK-Lu1	**DMSO**	85±5	90±11
"	**Dasatinib**	15±4	13±3
CALU-1	**DMSO**	96±9	100±10
"	**Dasatinib**	11±2	13±3
SW-900	**DMSO**	100±13	100±12
"	**Dasatinib**	12±2	14±3
CALU-6	**DMSO**	95±11	93±10
"	**Dasatinib**	8±2	10±3

[α]Cells were treated with 1μM Dasatinib, or the DMSO carrier when 50% confluent, for 24 hrs (see Methods).

[β] Stat3-tyr705 or Src-ptyr418 levels were measured by Western blotting, as in Table II, legend.

Table I
GJIC in nontransformed epithelial and lung cancer cell lines

	Cell line	Confluence[α]	GJIC[β]
Nontransformed epithelial	**E10**	90%	1.5±0.5
		100+3d	6±1
Lung cancer lines	**QU-DB**	90%	1±0.2
		100+3d	6.3±1
	SK-LuCi6	90%	1.8±0.2
		100+3d	6.5±1
	A549	90%	0.1±0.1
		100+3d	0.3±0.1
	SK-Lu1	100+3d	0.8±0.2
	CALU-1	100+3d	0
	SW-900	100+3d	0
	CALU-6	100+3d	0
	SK-LuCi6-*Src*	100+3d	0.2±0.1
Adenosquamous carcinoma, moderately differentiated	**carcinoma cells[γ]**	100+3d	0
	fibroblasts[γ]	100+3d	5.8±1.2
Adenocarcinoma, poorly differentiated	**carcinoma cells[γ]**	100+3d	0
Adenocarcinoma	**carcinoma cells[γ]**	100+3d	0

[α]Confluence was quantitated by imaging analysis (see Methods).

[β]GJIC was assessed by *in situ* electroporation at the indicated confluence (see Methods, Fig. 6). Quantitation was achieved by dividing the number of cells into which the dye had transferred through gap junctions (denoted by dots, Fig. 1B and 2A), by the number of cells at the edge of the electroporated area (denoted by stars). Numbers are averages ±SEM of at least three experiments, where transfer from more than 200 cells was examined.

[γ]Immediately after surgery, cells were placed in culture and GJIC examined (see Methods, Fig. 5). After 8-10 weeks in culture, most of the tumor cells had died while the fibroblasts present in the initial suspension predominated. These cells did not express cytokeratins, contrary to tumor cells [18]. The fibroblasts shown were derived from the moderately differentiated adenosquamous carcinoma tumor above (Fig. 3, *c-d*).

JUNK
(Old Table I)

Table I
GJIC in nontransformed epithelial and lung cancer cell lines

	Cell line	Confluence[α]	GJIC[β]
Nontransformed epithelial	**E10**	90%	1.5±0.5
		100+3d	6±1
Lung cancer lines	**QU-DB**	90%	1±0.2
		100+3d	6.3±1
	SK-LuCi6	90%	1.8±0.2
		100+3d	6.5±1
	A549	90%	0.1±0.1
		100+3d	0.3±0.1
	SK-Lu1	100+3d	0.8.0±0.2
	CALU-1	100+3d	0
	SW-900	100+3d	0
	CALU-6	100+3d	0
	SK-LuCi6-*Src*	100+3d	0.2±0.1
Adenosquamous carcinoma, moderately differentiated	**carcinoma cells[γ]**	100+3d	0
	fibroblasts[γ]	100+3d	5.8±1.2
Adenocarcinoma, poorly differentiated	**carcinoma cells[γ]**	100+3d	0
Adenocarcinoma	**carcinoma cells[γ]**	100+3d	0

[α]Confluence was quantitated by imaging analysis (see Methods).

[β]GJIC was assessed by *in situ* electroporation at the indicated confluence (see Methods, Fig. 6). Quantitation was achieved by dividing the number of cells into which the dye had transferred through gap junctions (denoted by dots, Fig. 1B and 2A), by the number of cells at the edge of the electroporated area (denoted by stars). Numbers are averages ±SEM of at least three experiments, where transfer from more than 200 cells was examined.

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fibroblasts shown were derived from the moderately differentiated adenosquamous carcinoma tumor above (Fig. 3, *c-d*).

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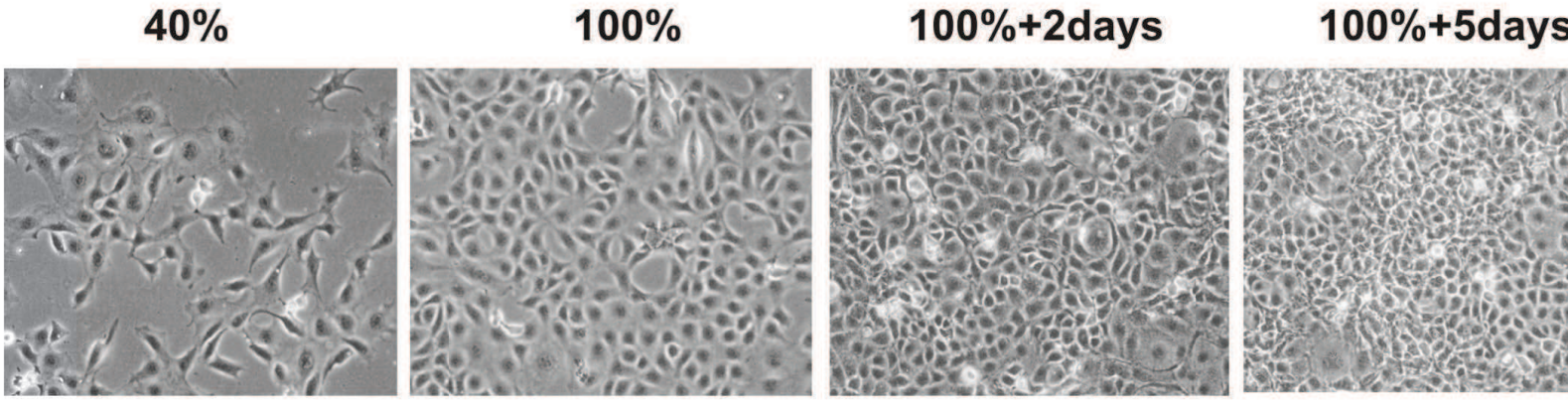
Figure 1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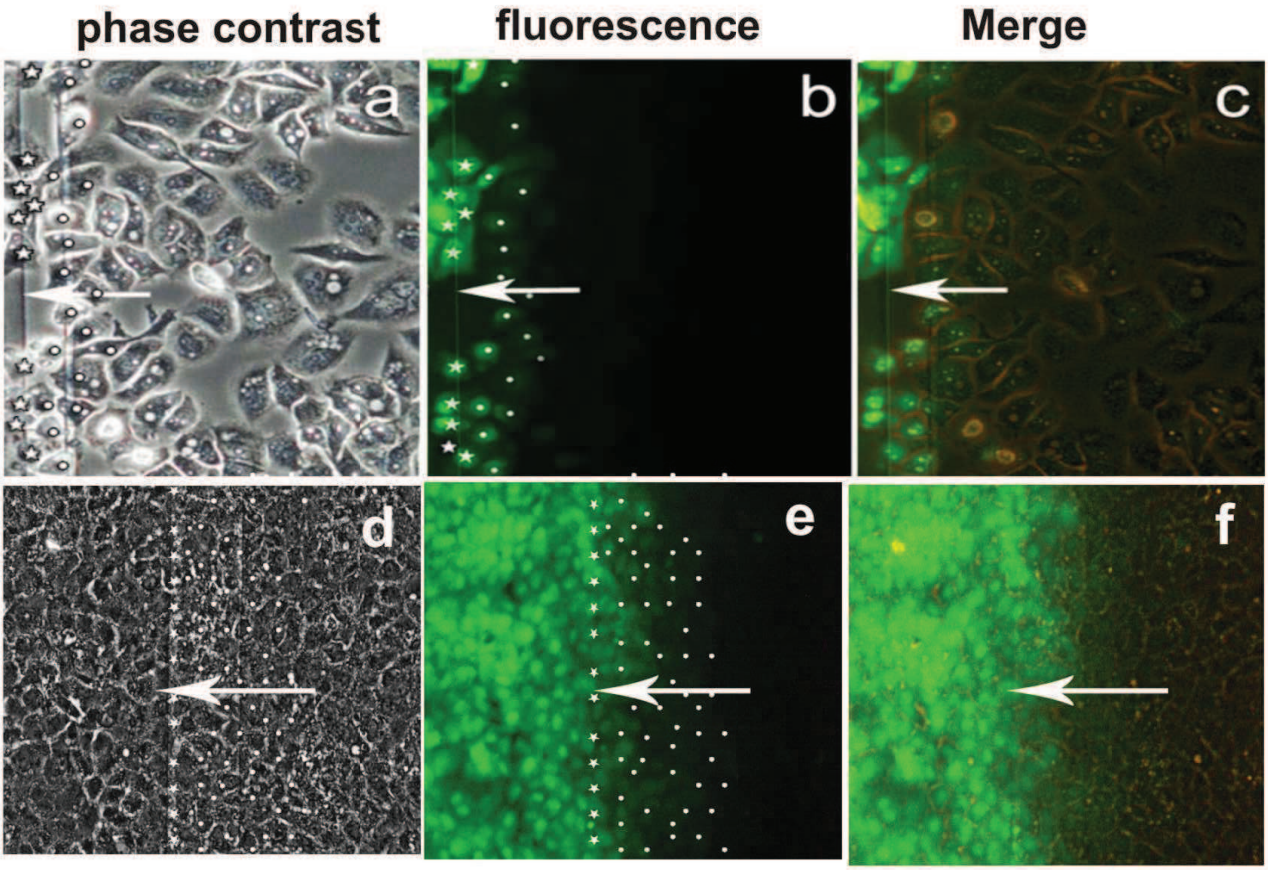
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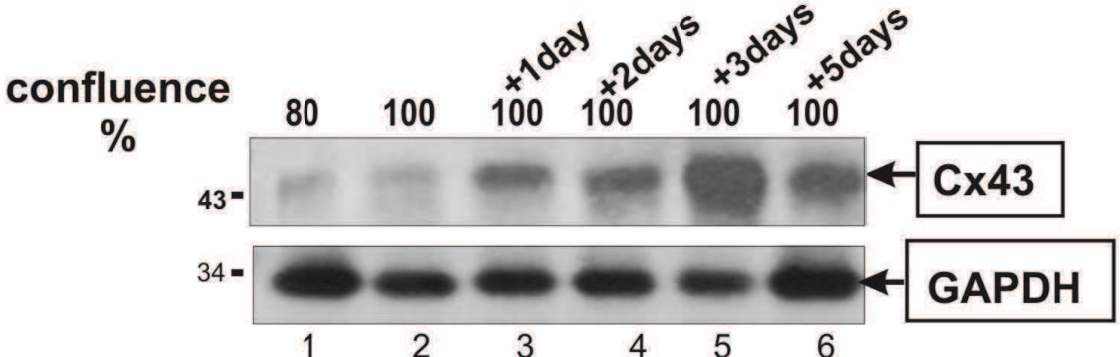
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Figure 2

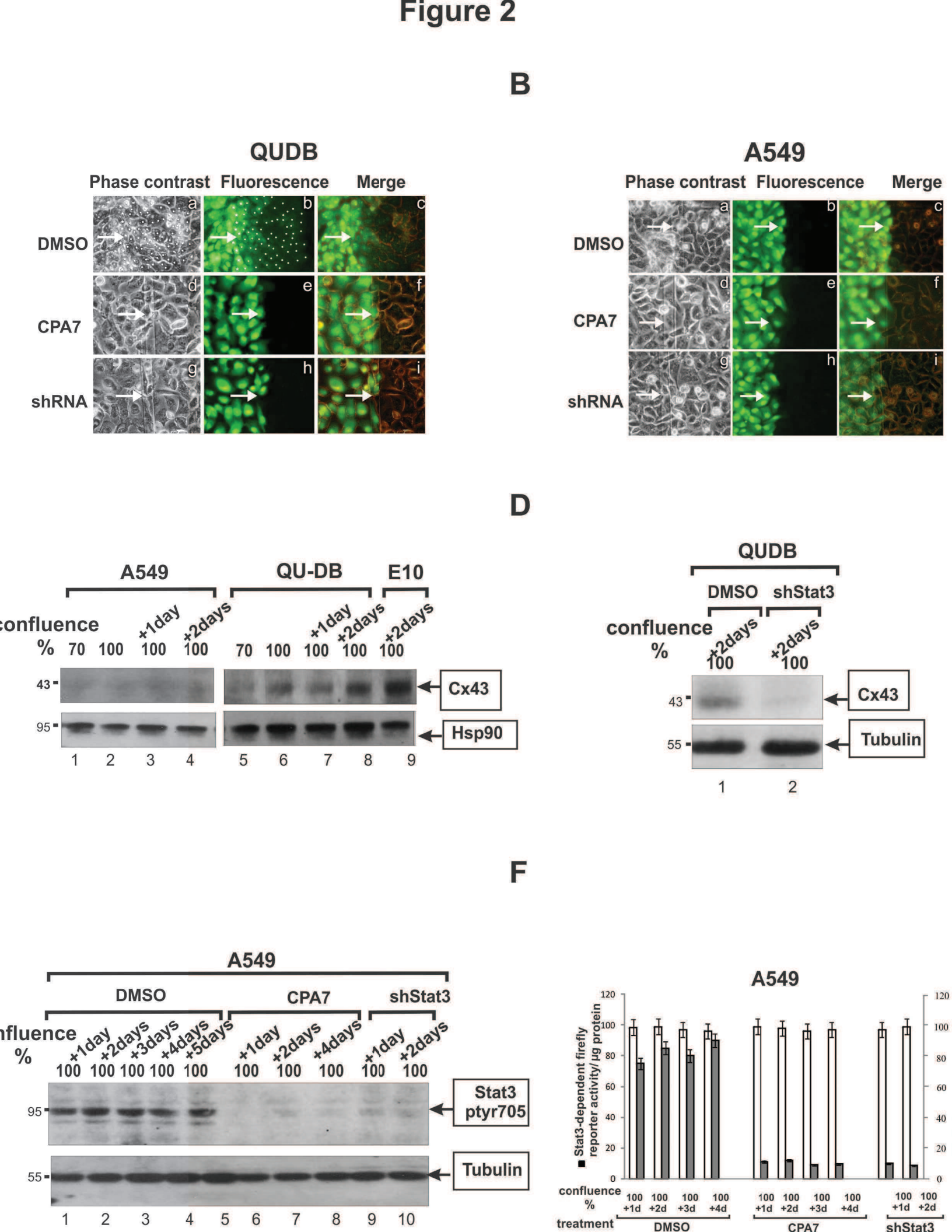
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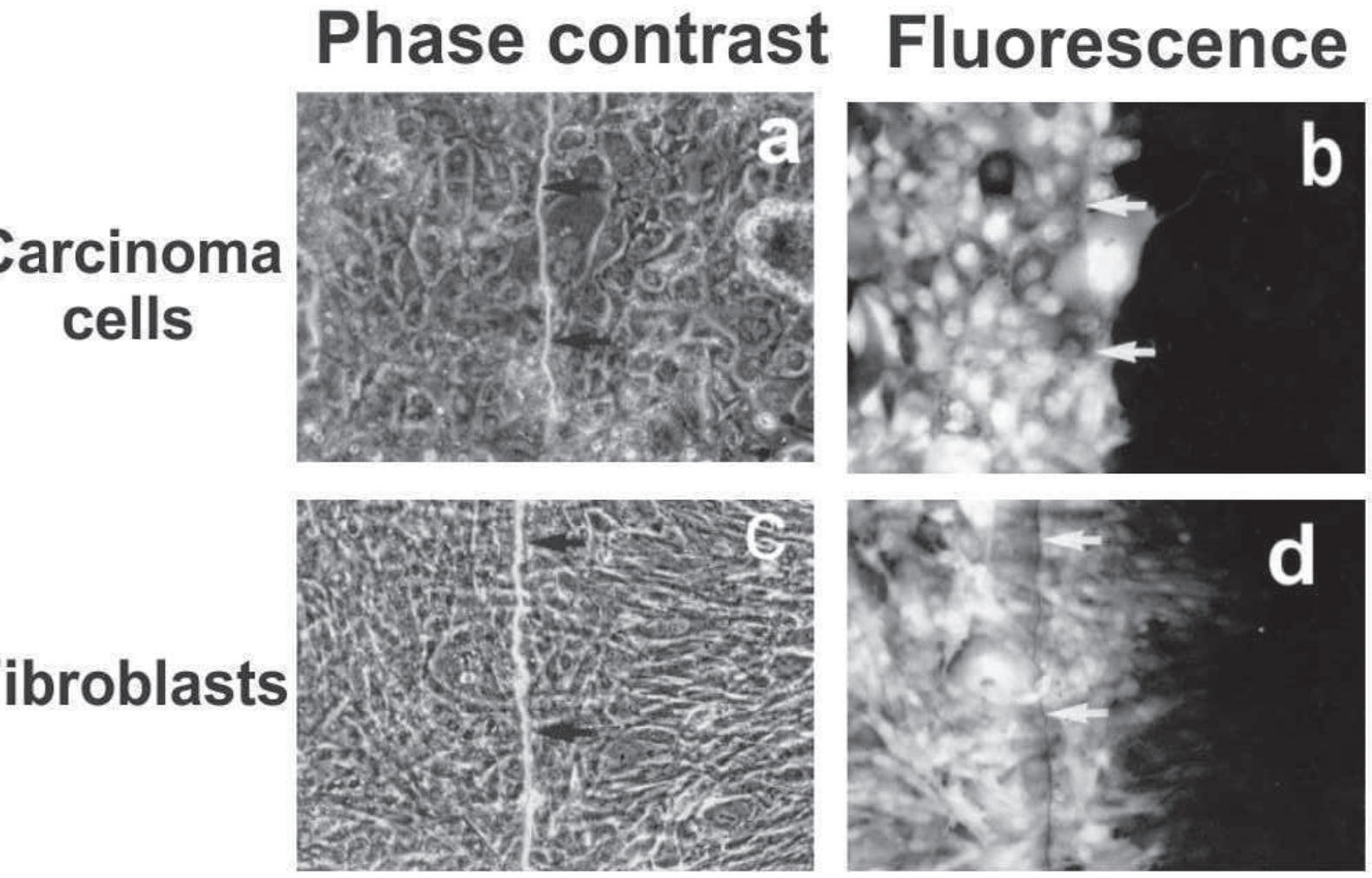
Figure 3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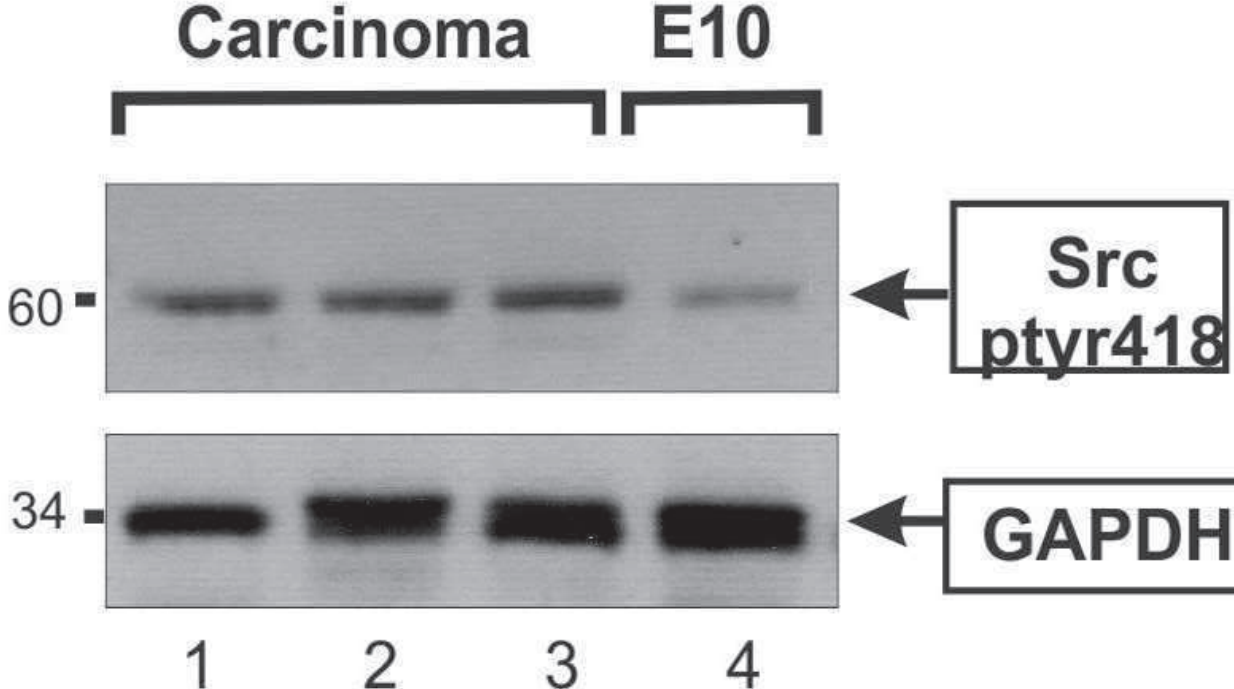
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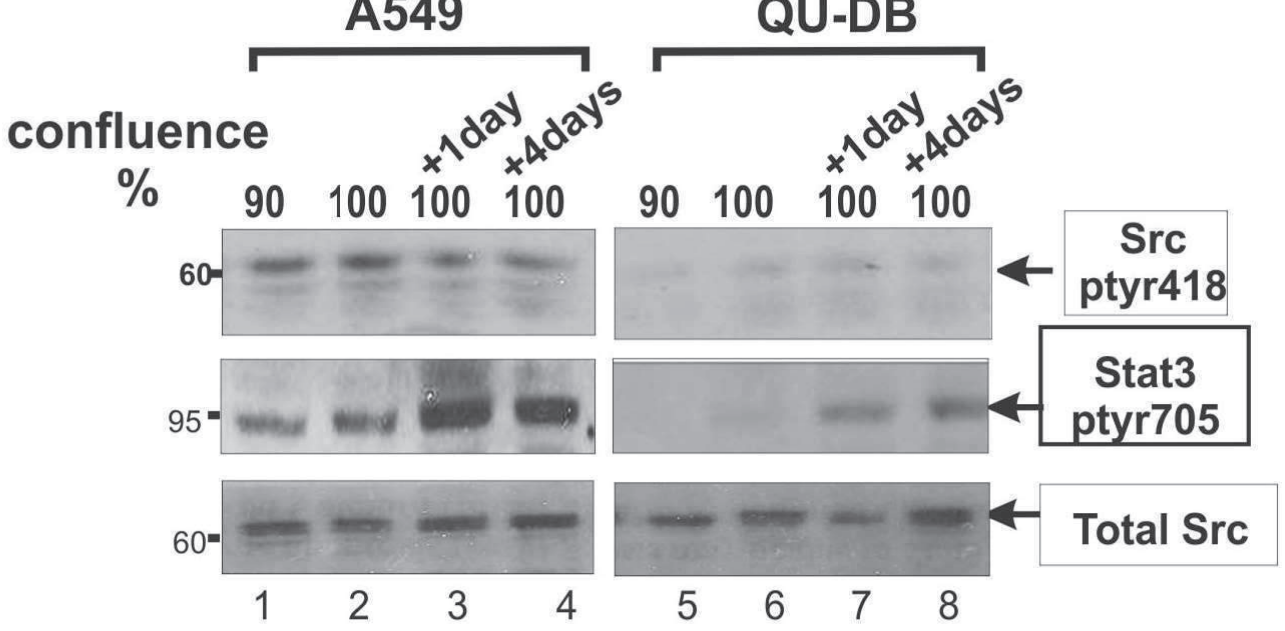
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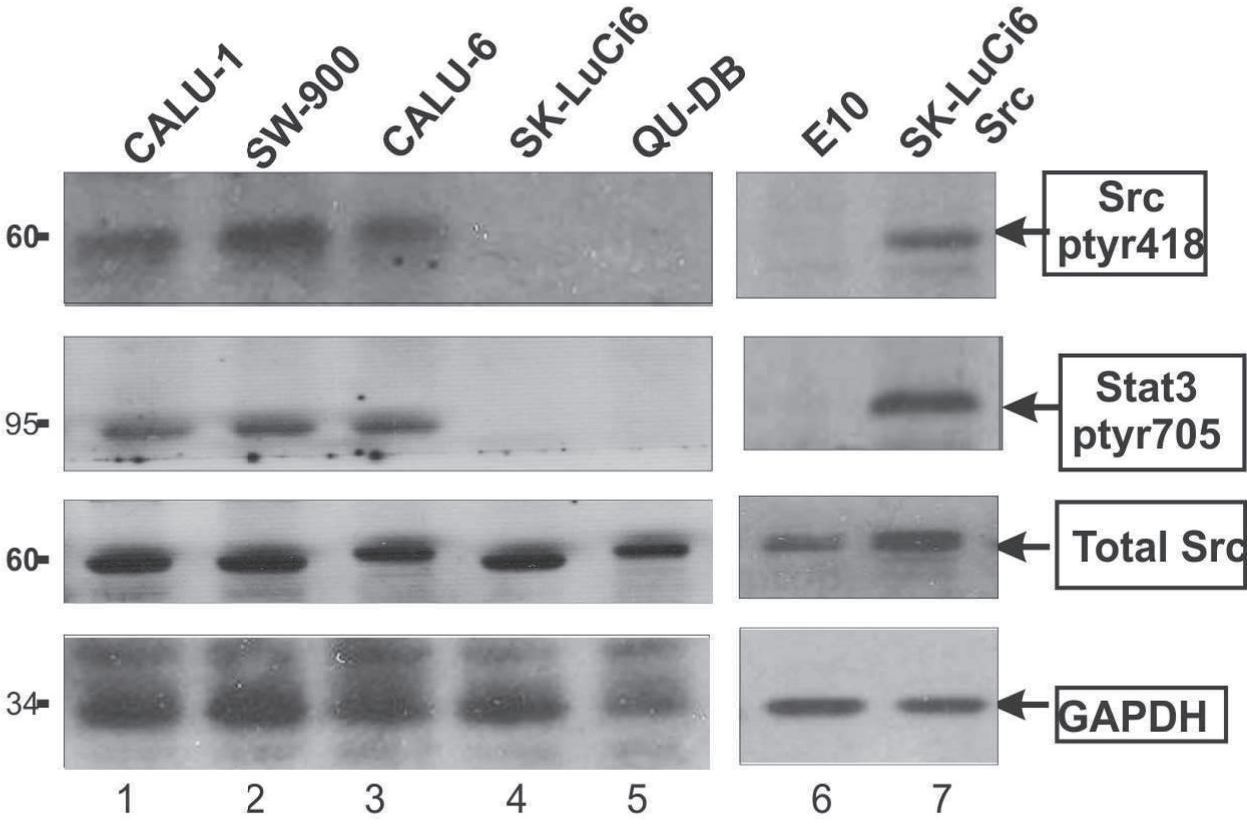
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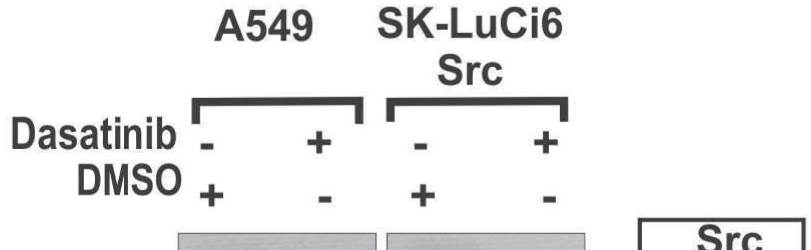
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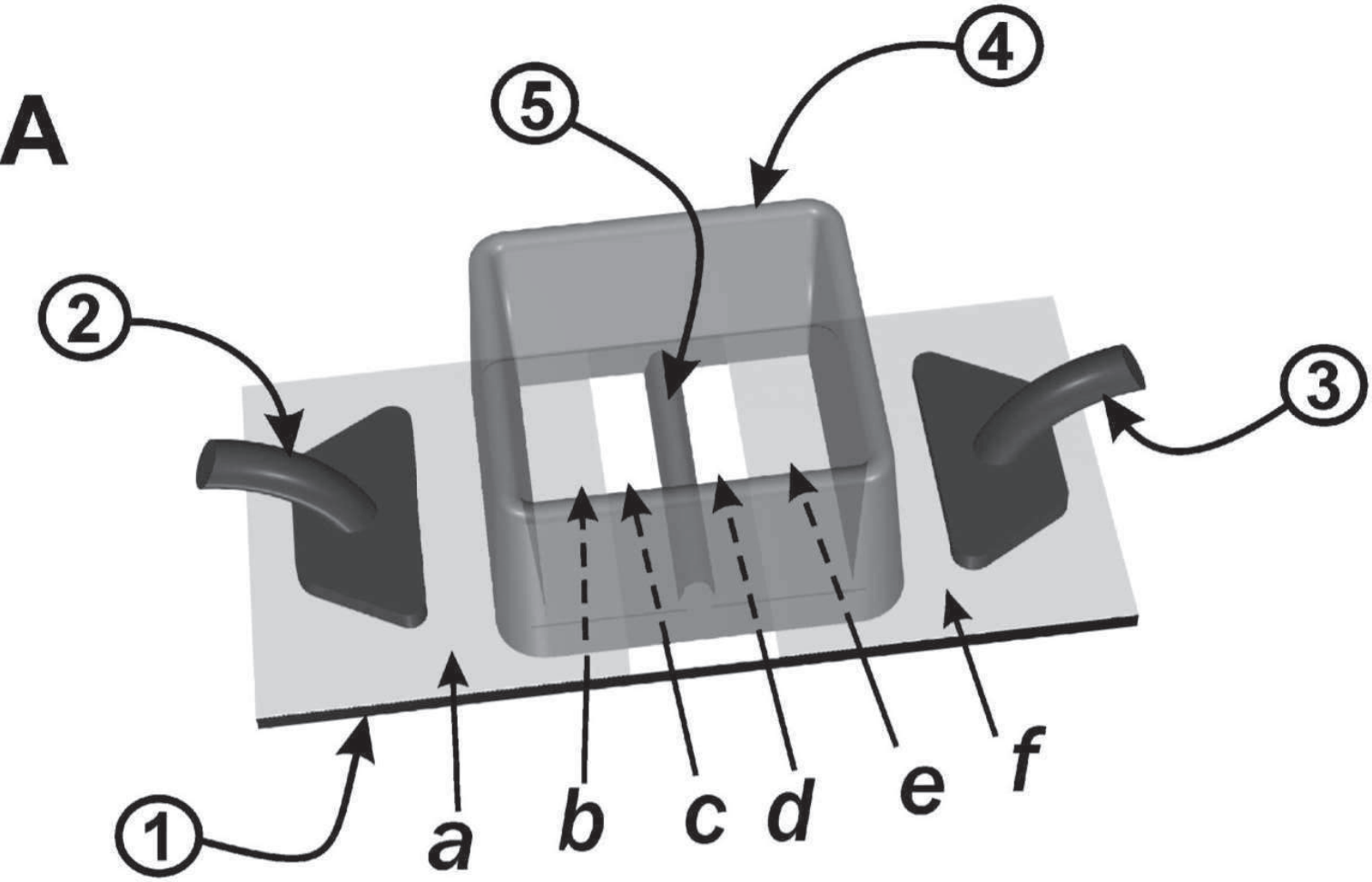
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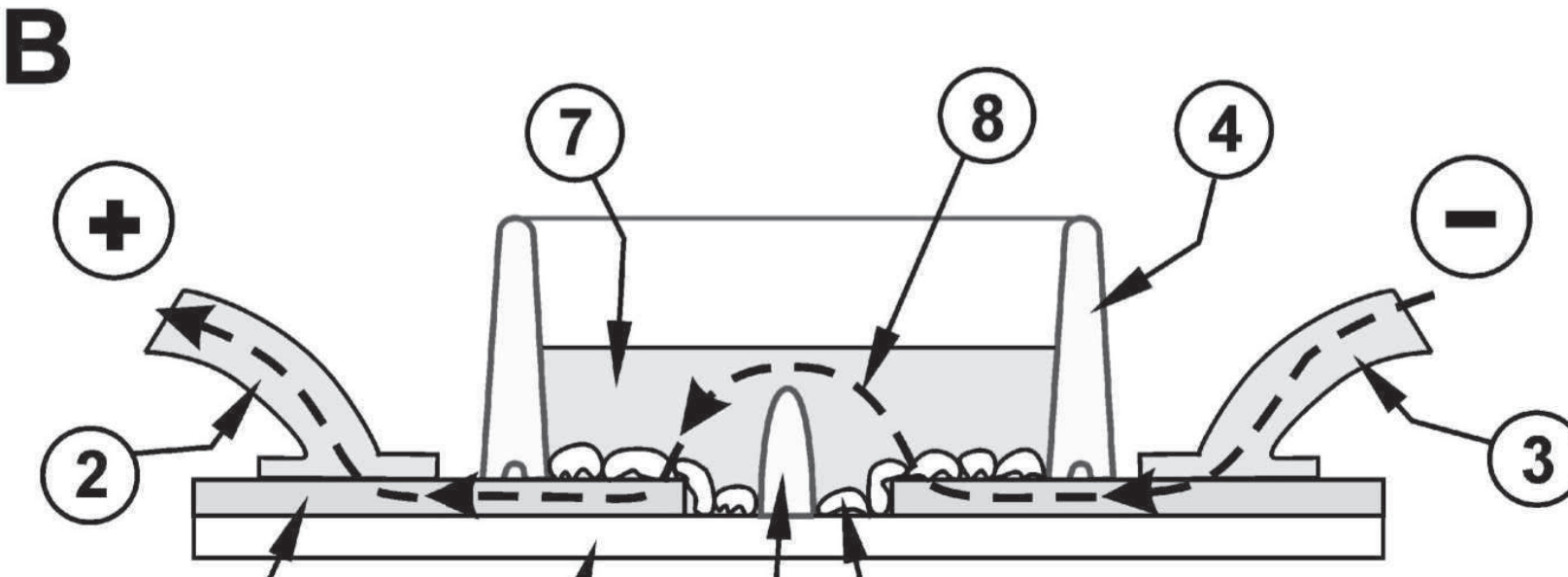
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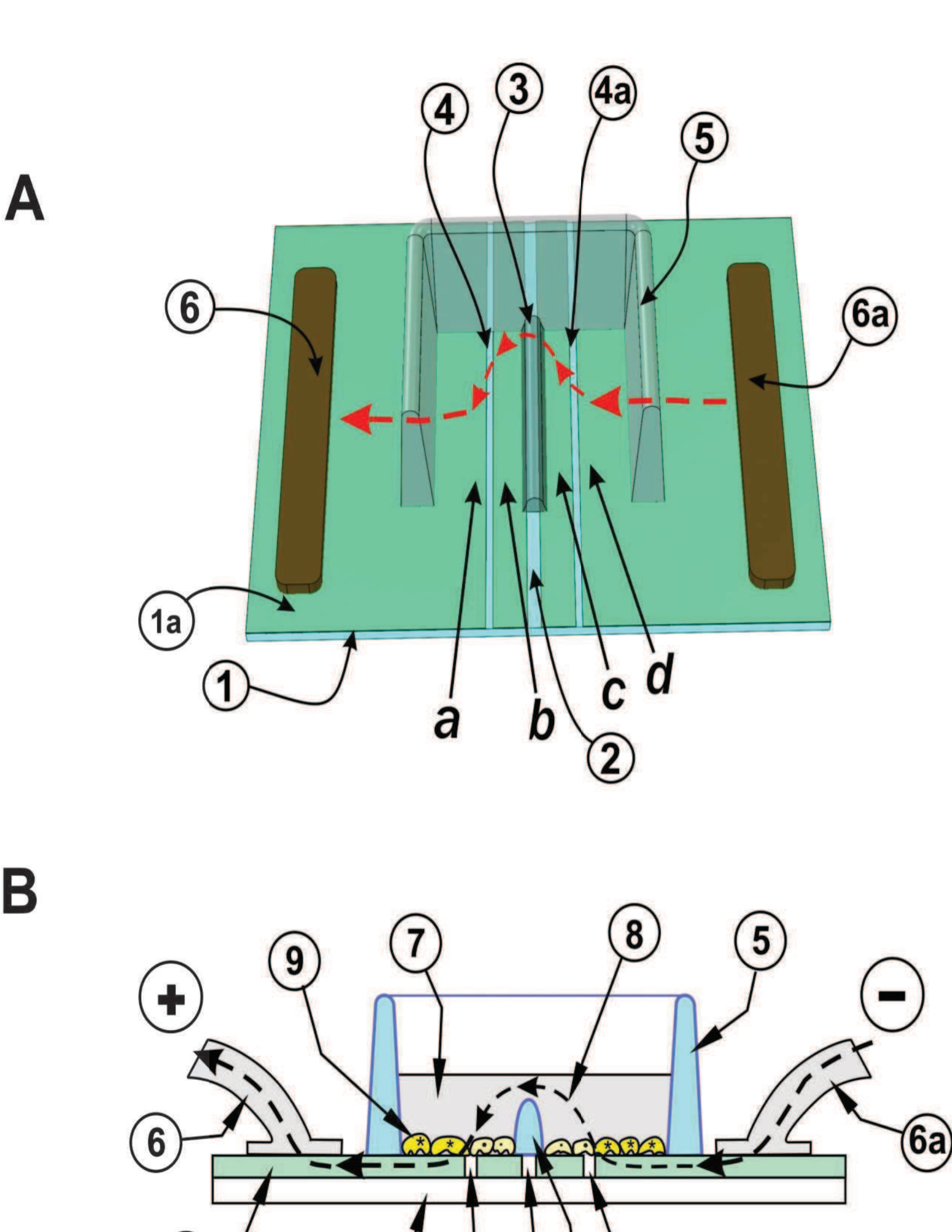
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Mini Review

Mind the Gap; Regulation of Gap Junctional, Intercellular Communication by the Src Oncogene Product and its Effectors

MULU GELETU, AARON TROTMAN-GRANT and LEDA RAPTIS*

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Abstract. *Gap junctions are channels that connect the interiors of neighboring cells and are formed by the connexin (Cx) proteins. A reduction in gap junctional, intercellular communication (GJIC) often correlates with increased growth and neoplastic transformation. Cx43 is a widely expressed connexin which can be phosphorylated by the Src oncoprotein tyrosine kinase on tyr247 and 265 and this reduces communication. However, Src activates multiple signalling pathways such as the Ras/Raf/Erk and PLCγ/protein kinase C, which can also phosphorylate Cx43 and interrupt communication. In addition, the Src effector Cas which has an adaptor function, binds Cx43 to suppress gap junctional communication. In sharp contrast, activation of a different Src effector, the cytoplasmic transcription factor Signal transducer and activator of transcription-3 (Stat3) is not required for the Src-mediated, GJIC suppression. In fact, Stat3 is actually required for the maintenance of gap junctional communication in normal cells with high GJIC.*

Contrary to unicellular organisms, cells in multicellular metazoa must divide under strict control. Thus, intercellular communication is crucial in the regulation of cellular functions and it often occurs indirectly through the release of diffusible growth factors by certain cells that initiate the signal through receptors on target cells. Communication between cells can also be achieved directly, through the gap junctions, *i.e.* channels running through the membrane which allow the passage of ions and other molecules between the interiors of adjacent cells. Gap junctions consist of the transmembrane proteins, termed connexins, a family of at least 20 members described in mammals. They are often designated with a suffix referring to their molecular weight. Gap junctions are formed by the aggregation of two hemi-channels of six connexons each, contributed by the two neighboring cells. This structure forms an aqueous channel through the two plasma membranes, that permits the passage of small molecules such as ions, nucleotides, aminoacids, short peptides or even RNA (24) between adjacent cells (46).

Results from a number of labs indicated that an increase in cell proliferation correlates with a reduction in gap junctional, intercellular communication (GJIC). In fact, a number of oncogene products such as the transforming protein of the Rous Sarcoma virus, vSrc (29), the polyoma virus middle Tumor antigen (mT (4, 35)), the activated chaperone Hsp90N (18), vRas (3, 8), tumor promotors such as the 12-O-Tetradecanoylphorbol-13-acetate and others have been shown to interrupt junctional communication.

src is an oncogene with a high clinical relevance and one of the best-studied targets for cancer therapy (reviewed in (1)). src encodes a potent oncoprotein with high tyrosine kinase activity (Src). Src can affect the activity of Cx43 by multiple mechanisms, namely by direct phosphorylation on tyrosine residues, but also by its direct downstream effector kinase pathways, Ras/Raf/Erk and the phosphatidylinositol-3 kinase (PI3k)/Akt that phosphorylate Cx43 on serine residues. In addition, Src may indirectly activate the ser/thr kinase, protein kinase C that can phosphorylate Cx43 and block gap junctions, as well as other kinases (5,26,33,38). Besides activating kinase pathways, Src can make use of the adaptor protein Cas (Crk associated substrate) that binds Cx43 to suppress gap junctional communication (39). Src is also a potent activator of the cytoplasmic transcription factor, Signal transducer and activator of transcription-3 (Stat3). In this communication we review the prevailing evidence on the role of Src and its effector pathways upon Cx43 and GJIC.

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Key Words:

Phosphorylation of Cx43 on tyrosine by the Src kinase

A reduction in gap junctional communication of Src-transformed cells was reported for the first time in 1966 (31). Subsequent cloning of Cx43 enabled a molecular characterisation of the mechanism whereby Src affects Cx43 function, and this led to fundamental studies on Cx43 regulation. A combination of genetic and biochemical evidence indicated that Src can phosphorylate Cx43 directly: At first the SH3 domain of Src binds a proline-rich area between P274 and P284 of Cx43. This brings the Src kinase domain in close proximity to Y265, which is then phosphorylated by Src (Figure 1). The phosphorylated Y265 offers a docking site for the Src, Src-homology-2 (SH2) domain and this enhanced interaction causes the phosphorylation of Y247 of Cx43, which may contribute to GJIC reduction (10,30,43). In fact, vSrc co-expression with a Cx43 mutant where tyr247 and tyr265 were replaced by phenylalanine in Cx43 knockout cells, was unable to interrupt communication, indicating that tyr247 and ty265 are important for GJIC suppression by the Src kinase (30). However, expression of the same Cx43 mutants in Xenopus oocytes can result in the formation of gap junctions, but these gap junctions can be disrupted by Src, indicating that the sites of direct phosphorylation by Src are not required for GJIC suppression in this setting (29). This led to the hypothesis that Src effectors may play a role upon GJIC suppression.

Effect of Src effectors upon GJIC suppression

The Ras/Raf/Erk pathway. Prominent among the signalling cascades initiated by the Src kinase to effect neoplastic conversion is the Ras/Raf/Mek/Erk. Src activates the Ras GTPase, which triggers the translocation of the ser/thr kinase Raf to the membrane, leading to Raf activation. Raf then activates Mek, a dual-specificity kinase, which, in turn, activates Erk (37), through phosphorylation on both thr and tyr in a ^{P}TE^{P}Y motif. Activated Ras was shown to suppress GJIC (8), and Ras function is also required by mT, an oncogene which induces neoplastic conversion by binding to and activating cSrc, to reduce gap junctional communication (9).

Erk can phosphorylate Cx43 at S255, S279 and S282 (50). In fact, expression of Cx43 mutants with all Erk phosphorylation sites mutated to alanine induces gap junction formation, but these gap junctions are not disrupted by Src expression, indicating that phosphorylation by Erk is important for GJIC suppression by vSrc. In addition, pharmacological inhibition of Erk eliminates Src's ability to interrupt gap junctional communication (54) and it was recently shown that ser residues of Cx43 were phosphorylated in vSrc transformed cells (40). Taken together, these data suggest that Erk activation by Src may be important in GJIC suppression. However, in addition to the direct phosphorylation of Cx43 by the Erk kinase, Ras may also act through other effectors to downregulate GJIC, such as RalGDS, p120GAP, AG6 and others (22).

PI3k/Akt. One of the Src downstream effectors is the phosphatidylinositol-3 (PI3) kinase. Work from a number of laboratories has shown that activated Src activates class I, PI3K, which phosphorylates phosphatidylinositol-4,5-bisphosphate (PIP2) and phosphatidylinositol-4-phosphate (PIP), to generate phosphatidylinositol-3,4,5-trisphosphate (PIP3) and PI(3,4)P2 respectively, in a reaction which can be reversed by the tumor suppressor PIP3 phosphatase, PTEN. The ser/thr kinase Akt binds to PIP3 at the membrane, by virtue of an amino-terminal plekstrin homology domain. Then the PDK1 kinase, also bound to PIP3 at the membrane, phosphorylates the activation loop of Akt at thr308 (Akt1 numbering). Another complex activated by RTK's, the mammalian target of rapamycin complex-2 (mTORC2) phosphorylates Akt1 on the carboxylterminal, hydrophobic domain, at ser473. Akt is thus transiently localised to the plasma membrane during activation and once activated, it phosphorylates substrates throughout the cell to regulate multiple cellular functions, including growth modulation, survival, proliferation and metabolism. Three isoforms have been described, Akt1, Akt2, Akt3, and studies from knockout mice documented distinct functions for each isoform (reviewed in (16,32)).

The effect of Src-mediated, PI3K/Akt activation upon GJIC is complex. Akt1 was shown to phosphorylate Cx43 at ser373 and ser369 (34). It was recently demonstrated that Akt is essential for the disruption of gap junctional communication by Src, while the expression of a constitutively active Akt1, but not Akt2 or Akt3 was sufficient to suppress GJIC in rat fibroblasts (21). However, results from osteoblasts indicated that PI3K/Akt is necessary for the maintenance of the steady-state expression of Cx43 through an effect on post-transcriptional mRNA stability (6). Given the large variety of substrates of the different Akt isoforms, it is possible that the effect of Akt activation by Src may be different in different settings.

PKC. Protein kinase C (PKC) is a Src effector serine/threonine kinase (17). Src activates PKC through activation of phospholipase Cγ, but also through direct phosphorylation (19). PKC phosphorylates Cx43 at S368 and S372 (12,27,28). These phosphorylation events reduce coupling, since PKC activation with 12-OTetradecanoylphorbol-13-Acetate (TPA) leads to a reduction in GJIC (5, 26). Inhibition experiments indicated that the PKCγ isoform reduces GJIC in lens epithelial cells (49), while PKCα,β or δ can disrupt coupling between fibroblasts (11).

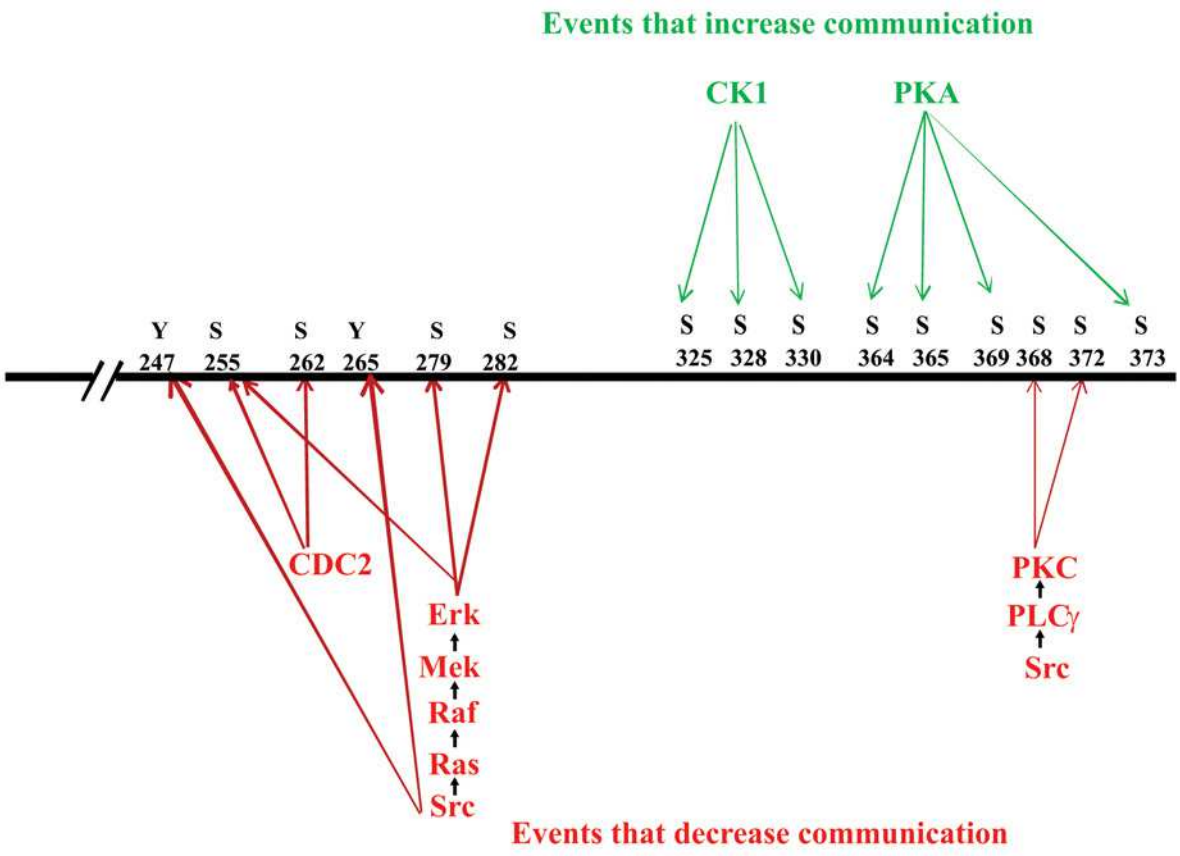

Figure 1. *Cx43 phosphorylations that affect gap junctional communication. Cx43 is shown with the phosphorylation sites targeted by Src and its effector kinases, Ras/Erk, and PKC, by aminoacid number and phosphorylating kinase. Sites phosphorylated by other kinases, such as protein kinase A (PKA) and caseine kinase 1 (Ck1) that increase communication, and the cell division cycle-2 ($p34^{Cdc2}$) kinase that decreases it are also indicated (23, 25, 44). Although the interplay between Src and these kinases has not been firmly established, it is possible that in addition to activating kinases that inhibit GJIC, Src may also suppress the activity of kinases that are required for communication (33). In red are events that decrease communication, in green events that increase it.*

Stat3. The signal transducer and activator of transcription-3 (Stat3) is latent in the cytoplasm in unstimulated cells and is activated by cytokine receptors of the IL6 family, as well as by tyrosine kinase receptors such as EGF-R and PDGF-R (52). Ligand-induced assembly of cell surface receptor complexes causes receptor activation. Subsequent tyrosine phosphorylation of the receptor cytoplasmic tail by the receptor itself or by the associated Jak or Src tyrosine kinases, creates docking sites for recruiting latent, unphosphorylated Stat3 *via* its Src homology 2 (SH2) domain. The receptor-bound Stat3 becomes a substrate for phosphorylation at a critical tyrosine (tyr705). This activates Stat3 by stabilizing the association of two monomers through reciprocal SH2-phosphotyrosine interactions. The Stat3 dimer then binds to specific target sequences in the nucleus, leading to the transcriptional activation of genes which play a role in cell proliferation and survival, such as myc, cyclin D, Bcl-xL, survivin, Hepatocyte Growth Factor (20), Vascular Endothelial Growth Factor and others (13, 52). Activated Src also activates Stat3 and this is required for Src transformation. It was shown that the process requires the activity of the Jak1 kinase as well, while wild-type or kinase-inactive PDGF-receptor enhances Stat3 activation by vSrc, serving a scaffolding function (53). Stat3 is found to be hyperactive in a number of cancers (15), and the fact that a constitutively active form of Stat3 alone is sufficient to induce neoplastic transformation (7) points to an etiological role for Stat3 in neoplasia.

Stat3 down-regulation does not restore GJIC in Src transformed cells. Previous results from our lab and others demonstrated that engagement of E-cadherin, as brought about by confluence of adherent, cultured cells causes a dramatic increase in Stat3, ptyr705 phosphorylation and activity (41, 42, 47, 48), therefore density must be taken into account when assessing the effect of inhibitors upon Stat3 activity. To

examine the effect of Stat3 down-regulation upon GJIC, Stat3 activity was reduced using the pharmacological inhibitor CPA7 (2), or infection with a retroviral vector carrying a Stat3-specific, siRNA (14). For these experiments, GJIC was examined using an apparatus of electroporation in situ, on a partly conductive slide (36). The fluorescent dye, Lucifer yellow, was electroporated into cells grown on electrically conductive, optically transparent, indium-tin oxide, followed by observation of the migration of the dye to the adjacent, nonelectroporated cells under fluorescence illumination. The results demonstrated that, contrary to inhibition of the Ras/Erk pathway, Stat3 inhibition in cells expressing activated Src does not restore GJIC, indicating that Stat3 is not part of a pathway of Src-induced, GJIC suppression (14).

Stat3 is required for the maintenance of gap junctional communication in normal epithelial cells and fibroblasts. Since Stat3 knockdown did not restore GJIC in Src-expressing cells, the possibility that Stat3 might have a positive role upon GJIC was explored. In fact, Stat3 knockdown in normal rat liver epithelial T51B cells which have extensive GJIC (14), or certain lung cancer lines that retain GJIC (Geletu *et al*., in preparation), abolished junctional communication and caused a dramatic reduction in Cx43 levels. That is, rather than increasing communication, Stat3 inhibition eliminates GJIC, indicating that Stat3 activity is, in fact, required for the maintenance of gap junction function in normal cells with extensive GJIC. This could be related to Stat3's ability to prevent apoptosis, since apoptotic death induction through cycloheximide, etoposide or puromycin caused a rapid loss of coupling, due to caspase-3-mediated degradation of Cx43 (45). Whether a similar mechanism might apply to GJIC suppression following Stat3 inhibition remains to be determined. In any event, current evidence demonstrates that Stat3, although it is generally growth promoting and in an activated form can act as an oncogene, its function is actually required for the maintenance of junctional permeability.

Final remarks. Besides Src, a variety of oncogenes and growth factors are known to cause gap junction closure. Although the effects of neoplastic transformation upon GJIC are clear, the mechanisms whereby this leads to tumor growth and progression are not well defined. A better understanding of the relationship between Src and Cx43 will offer useful insights on the paths leading to carcinogenesis. Since Src also plays an important role in other cellular functions, this will also unveil mechanisms involved in processes such as development and homeostasis (51).

Acknowledgements

The financial support from the Canadian Institutes of Health Research, the Canadian Breast Cancer Foundation (Ontario Chapter), the Natural Sciences and Engineering Research Council of Canada, the Canadian Breast Cancer Research Alliance, the Ontario Centers of Excellence, the Breast Cancer Action Kingston and the Clare Nelson bequest fund through grants to LR is gratefully acknowledged. MG was supported by a postdoctoral fellowship from the US army Breast Cancer Program (#BC087586), the Ministry of Research and Innovation of the Province of Ontario and the Advisory Research Committee of Queen's University.

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Received July 24, 2012
Accepted August 20, 2012

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International Journal of Cancer Research and Treatment

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